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13. ABSTRACT (Maximum 200 Words)

Development of anti-HER-2 therapy (trastuzumab) has been a major advance in breast cancer. The role of tissue and blood biomarkers in predicting response to trastuzumab is relatively unexplored. Our study shows no significant correlation with HER2 copy number and response (mean 6.25 vs 5.6 copies/Ch17). We postulate that once selected for HER2 amplification, copy number does not influence response to therapy. HER2 extracellular domain (ECD) was present in 24/47 (51%) HER2+ metastatic breast cancer patients and correlated with visceral disease (p=0.018). ECD levels declined in all patients receiving trastuzumab, however the slope of decline in was shallower in progressing patients (ROC 0.89). We conclude that the utility of this biomarker in predicting response to trastuzumab therapy remains to be established. Circulating tumor cells (CTCs) were found in 44% of HER2 positive metastatic breast cancer patients by quantitative real-time RT-PCR for Cytokeratin 19 and were associated with liver metastases (p=0.00006). CK-19 declined in all responding patients and became elevated in 5/15 (30%) of progressing patients. All patients with HER-2-ECD >50ng/ml had CK-19 cells detectable during therapy (p=0.008). Quantitative RT-PCR for HER2 in CTCs showed much lower sensitivity and specificity compared with CK19, and was not found to be a useful measure in tracking disease.

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Introduction

The *c-erbB-2* protooncogene codes for a 185 kDa receptor tyrosine kinase (HER-2, ErbB2) which is overexpressed in 30% of invasive breast cancer and correlates with a worse prognosis[i]. The extracellular domain (ECD) of the HER-2 receptor has been detected in the serum of 21%-25% of invasive breast cancer patients with 91-100% of these tumors overexpressing HER-2[ii]. Elevated levels of HER-2/ECD are only detected in patients with tissue expression of HER-2, suggesting that this may be a useful circulating marker for HER-2 disease detection and response to therapy[iii] [iv]. In addition, newer detection methods allow the detection of circulating tumor cells (CTCs) in peripheral blood. The correlation between serum HER-2 expression and detection of circulating tumor cells is not known.

The humanized monoclonal antibody, trastuzumab, is now standard of care for metastatic breast cancer patients with HER-2 positive tumors[v]. The role of circulating biomarkers in predicting response to trastuzumab-based therapy is unclear. Dr Raquel Andrade Nunes (postdoctoral award recipient) and Dr. Cinara Dias (subsequent postdoctoral fellow), have studied these questions in the laboratory of Dr. Lyndsay Harris during the period of this award. The following report summarizes their accomplishments and reportable outcomes:

Body

Aim 1: To evaluate the utility of measuring circulating HER2 Extracellular Domain (ECD) in Metastatic Breast Cancer Patients in monitoring Response and Progression of Trastuzumab® and Chemotherapy.

During the period of this award, Dr. Nunes and subsequently Dr. Dias worked closely with the data managers in Dr. Harris' laboratory and clinical research coordinators to ensure that samples were collected and processed for patients and normal donors who donated blood specimens for this study. The following summarizes patients/samples accrued:

- 55 patients with metastatic breast cancer from 16 medical institutions
- 287 blood samples have been collected on these 55 patients.
- 49 patients with stage II and III HER2 positive breast cancer had 447 blood samples collected.
- Plasma from 40 healthy female donors from the DFCI were collected, processed and stored for later analysis.

Collection of blood for HER2/ECD and circulating tumor cells was performed using a special phlebotomy tube (cell preparation tube - CPT) that allowed plasma and peripheral blood mononuclear cells to be obtained from one blood sample. Samples were processed immediately upon receipt using density gradient separation. Plasma was collected and stored at –80C. Peripheral blood mononuclear cells were subjected to rate-controlled freezing in order to preserve cell viability and stored at –140C. All samples were analyzed in batches of 50-100.

HER2/ECD was measured using a commercially available kit (HER2/neu ELISA®; Oncogene Science). Samples to be analyzed were processed in triplicate with 5 internal controls to plot a standard curve in order to quantify the levels of HER2/ECD in the plasma. In order to protect the confidentiality of patients and integrity of the study, the data manager records information on patient samples while the study coordinators store clinical information in a separate research database. After biomarker measurement, these datasets were merged for statistical analysis by comparing rate frequency and level of expression of HER2 ECD with disease characteristics and response to therapy.

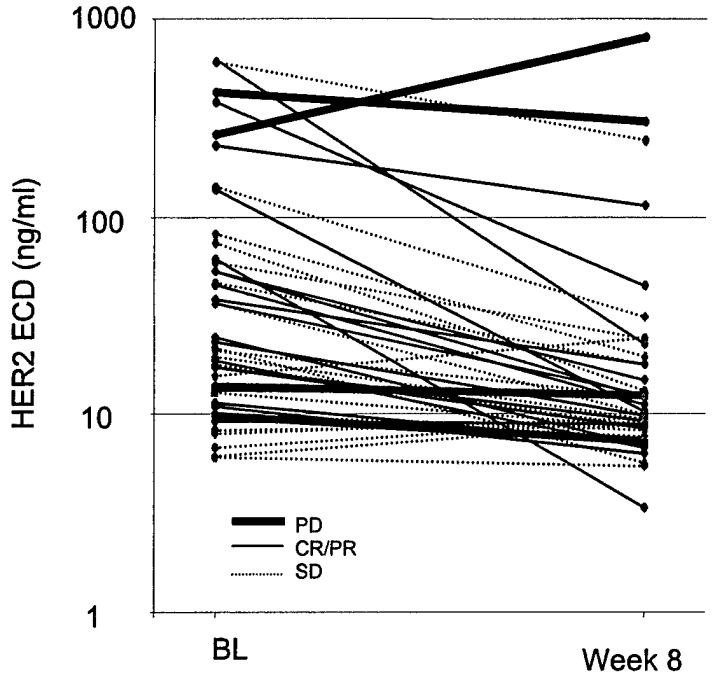
The following summarizes these analyses, and publications which have arisen from this work:

In metastatic breast cancer patients with HER2 3+/FISH+ tumors, treated with Trastuzumab and vinorelbine, 24/47 (51%) patients had elevated HER2/ECD using a cut-off value of 20 ng/ml. The expression of HER2/ECD was correlated with the presence of visceral disease (p=0.018). HER2/ECD

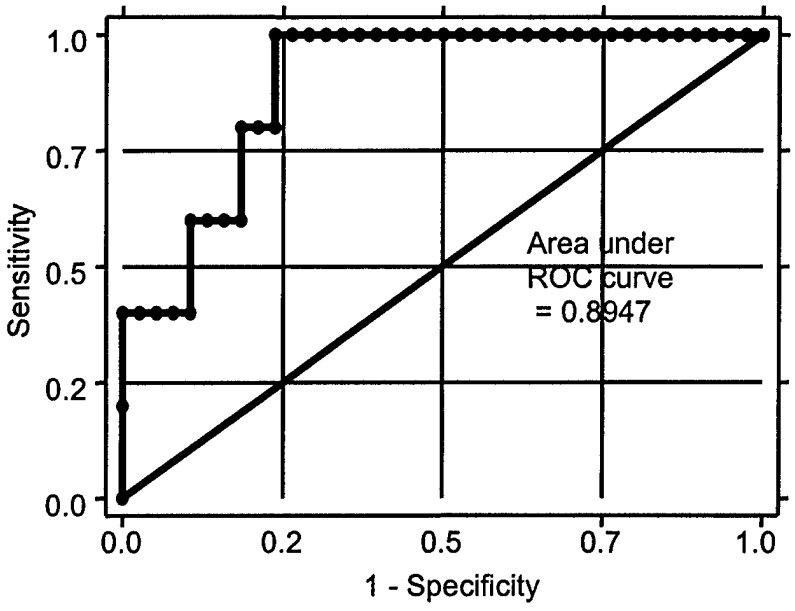
levels declined in both responding and progressing patients, however the percentage change between baseline and first cycle is lower in progressing patients (Fig 1, Burstein et al JCO 2003).

Figure 1. Serum HER2 ECD in Metastatic Patients Treated with Trastuzumab and Vinorelbine

Panel A. Change in HER2 ECD During Cycle 1 of Therapy



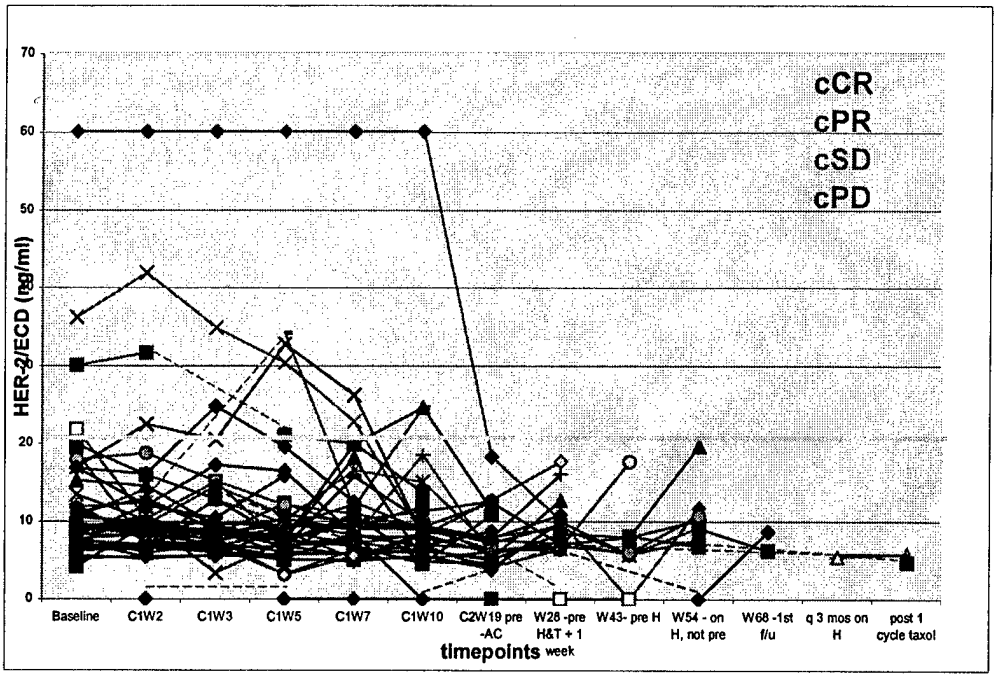
Panel B. ROC for PD with Change in Baseline HER2 ECD During Cycle 1



In early stage breast cancer patients (Stages II-III) treated with trastuzumab and vinorelbine,

- 4/45 patients had elevated HER2/ECD levels at baseline **(figure 2)**.
- 6/45 patients were non-responders; 2 of these had elevated HER2/ECD at baseline. Only one case had progressive disease and her HER2/ECD levels were never above baseline.
- It was not possible to establish a correlation between clinical response and HER2/ECD levels, given the limited sample size, although patients with a less favorable response (stable disease) appeared to have higher levels of ECD and a slower decline in response of the biomarker, as observed with advanced disease (Dias et al, European Society of Medical Oncology, 2003).

Figure 2: Serum HER2 in Early Stage Patients Treated with Trastuzumab and Vinorelbine



Aim 2: To compare methods of measuring HER2 in Breast Cancer Tissue

a) Coordinate retrieval of paraffin-embedded primary tumor tissue for patients enrolled in trastuzumab and chemotherapy trials at DFCI and participating institutions.

b) Perform immunohistochemistry and fluorescence in-situ hybridization on tumor tissue per methods outlined in proposal.

- Retrieval of tumor tissue collection from 91 patients, either as paraffin embedded blocks or unstained slides and the following assays were performed:
    - HER-2 protein expression was assessed by immunohistochemistry (IHC) in 91 patients in the Breast Cancer Core of the Dana-Farber Harvard Cancer Center, using the DAKO HercepTest® (Glostrup, Denmark).
    - HER-2 amplification was determined in 71 patients by fluorescent in situ hybridization (FISH) in a central laboratory using the PathVysion® kit (Vysis Inc., Downers Grove, IL), kit.

The following table summarizes results of these studies:

Table 1: HER-2 by IHC and FISH in Patients Treated with Trastuzumab and Chemotherapy

	IHC negative	IHC positive	IHC unknown	Total
FISH negative	**8**	**3**	**1**	**12**
FISH positive	**1**	**44**	**1**	**46**
FISH unknown	**10**	**14**	**9**	**33**
Total	**19**	**61**	**11**	**91**

As expected, the majority of FISH positive tumors were IHC positive for HER2. Of note, while gene amplification by FISH has been shown to be predictive of response to trastuzumab in other studies, comparison of gene copy number with response our cohort was not predictive (**Table 2**). While this observation may be due to small sample size, in patients selected for trastuzumab therapy by high-level expression of HER2, amplification and/or gene copy number may not add further value in predicting response to therapy – other molecular features of the tumor may modify response in this setting.

Table 2: Response to Trastuzumab-containing therapy and HER2 copy number by FISH

	Responders	Non-responders	total
metastatic cohort	15 patients	10	25
neoadjuvant	32	6	38
total	47	16	63

13+26=39 (83%) are FISH +
mean signal ratio is 6.25

7+6=13 (81%) are FISH +
mean ratio is 5.6

Aim 3: To detect and monitor the molecular phenotype of circulating tumor cells (CTC) in HER2 Positive Metastatic Breast Cancer patients treated with Trastuzumab® and Chemotherapy.

a) Organize the collection of peripheral blood samples from patients enrolled in trastuzumab and chemotherapy trials at DFCI and participating institutions.

b) Perform immunomagnetic selection and reverse transcription polymerase chain reaction (RT-PCR) for cytokeratin, BU-101 and HER2 on peripheral blood samples and compare with clinical parameters (disease burden, response to therapy).

Detection of CTCs using Quantitative RT-PCR for Cytokeratin 19

Positive selection of epithelial cells from the blood was performed using immunomagnetic beads (Dynabeads®) coated with anti-BER-EP4 antibody. Expression of cytokeratin 19 and HER2 gene products from malignant epithelial cells was measured by quantitative real-time RT-PCR. To establish the specificity of tumor cell detection, peripheral blood from 40 healthy female blood donors was collected from the Brigham and Women's Hospital Blood Donor Center, after informed consent. In 35 cases (87.5%), values of 10-50 c/s/s CK 19 transcripts were obtained in the duplicate analyses by LCx® Immunoassay (Abbott Laboratories). In one case, a value > 100 c/s/s was obtained in the duplicate analysis. In 4 cases, duplicate results were discordant (one value >100, one value <100 c/s/s) and a third analysis was performed. Based on these results, a positive result for CK-19 detection was defined as the presence of duplicate values >100 c/s/s. When the duplicates were discordant, values > 100 c/s/c in 2 out of 3 analyses were considered a positive result. B2MG was detectable in all cases. With this definition, CK-19 was detected in 2 (5 %) healthy controls, with counts averaging 479 c/s/s.

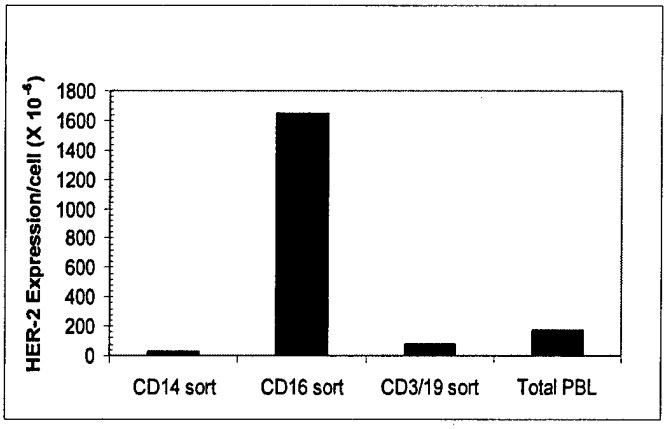
The sensitivity was established by spiking MDA-MB-361 (HER-2 amplified) cells into whole blood from healthy donors, collected in CPT and processed in a similar fashion to patient samples. All samples were analyzed in duplicate or in triplicate when results were discordant. Sensitivity was assessed by experiments where HER2 amplified cell lines were spiked into peripheral blood. In 20 such experiments, the presence of 10 MDA-MB-361 breast cancer cells spiked in 8 ml of blood was always detectable. CK-19 signal by LCx® consistently measured 900-1300 c/s/s for 10 breast cancer cells spiked into blood. RNA dilution assays allowed the detection of 0.01 cell equivalent of CK-19. No amplification was obtained with omission of the reverse transcription step, confirming that detection of genomic DNA did not occur. Data from this study in metastatic patients demonstrated the following salient points:

- CK-19 was detectable in 5% (2/40) of normal donors
- CK-19 was found in 44% of HER2 positive metastatic breast cancer patients prior to therapy.
- The presence of CK-19 before treatment was associated with liver metastases (p=0.00006).
- CK-19 signal declined in all responding patients after one or two cycles of therapy and became elevated in 5/15 (30%) of patients on progression.
- All patients with very high levels of serum HER-2 (>50ng/ml) had CK-19 cells detectable during therapy (p=0.008).

Analysis of HER2 in CTCs by Quantitative Real-time RT-PCR

The expression of HER-2 and B2MG genes was assessed by quantitative, real-time PCR using TaqMan® technology. The sensitivity of this method, based upon spiking HER2 amplified cells into peripheral blood, was considerably lower than that of CK19 (1 epithelial cell in 10^5 mononuclear cells) and variable expression of HER2 was seen in normal donors. Further study of normal donor peripheral blood samples suggested that the CD16 positive fraction (NK cells/granulocytes) were the major source of HER-2 signal (**figure 3**).

Figure 3: HER-2 and B2MG Quantitative RT-PCR results in peripheral blood leucocytes (PBL)



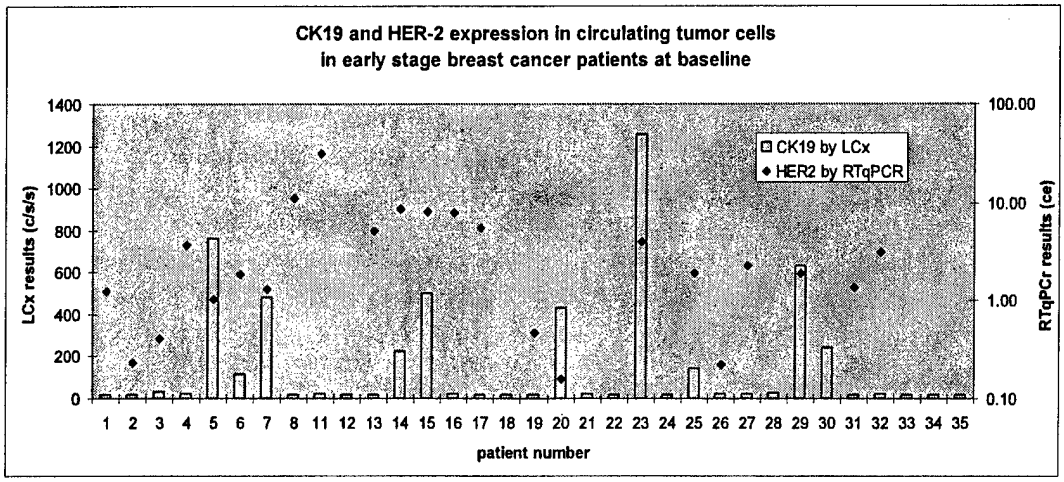


Figure 4. Correlation between CK19 and HER-2 expression in PBL of HER-2 positive breast cancer patients treated with Trastuzumab

In HER2 positive patient cohorts, assessment of HER2 in peripheral blood samples after immunomagnetic selection and quantitative real-time RT-PCR demonstrates no correlation with CK19, burden of disease or response to therapy (**Figure 4**). In fact, HER2 levels correlate best with beta-2 microglobulin levels suggesting that contaminating NK/granulocytes are the main source of signal in this assay (data not shown). We conclude that expression of HER2 in CTCs of patients with HER2 positive breast cancer is detectible above background levels, using this method. Other approaches (flow cytometry, immunohistochemical assays) may yield better sensitivity/specificity for HER2 in this setting. These data are part of a manuscript in preparation by Dr. Dias and colleagues.

Key Research Accomplishments

- Our tissue studies suggest that HER2 copy number by fluorescent in-situ hybridization does not correlate with response to trastuzumab containing-therapy.
- Our serum studies show that HER2/ECD is not a useful biomarker in predicting response in trastuzumab-treated patients.
- Our studies of circulating tumor cells by quantitative RT-PCR after immunomagetic selection show that CK19 by Quantitative RT-PCR is a useful marker for circulating tumor cells, which appear to track with disease burden and response to therapy. HER2 by real-time quantitative PCR lacked sensitivity and specificity necessary for clinical utility.

Reportable outcomes

Nunes R, Harris LN. The HER2 extracellular domain as a prognostic and predictive factor in breast cancer. Clin Breast Cancer 2002 Jun;3(2):125-35; discussion 136-7.

Burstein HJ, Harris LN, Gelman R, Lester SC, Nunes RA, Kaelin CM, Parker LM, Ellisen LW, Kuter I, Gadd MA, Christian RL, Kennedy PR, Borges VF, Bunnell CA, Younger J, Smith BL, Winer EP. Preoperative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II or III breast cancer: a pilot study. J Clin Oncol. 2003 Jan 1;21(1):46-53.

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Raquel A Nunes, Taylor Ngo, Madhavi Kamma, Lisa Roberts, Paula Friedman, Natalie Shilo, Walter Carney, Harold Burstein, Kimberly Blackwell, Paula Ryan, Virginia Borges, Eric P. Winer, J. Dirk Iglehart, Lyndsay N. Harris. Circulating Tumor Cells in HER2 Positive Metastatic Breast Cancer Patients treated with Trastuzumab and Chemotherapy. submitted to Clinical Cancer Research.

Conclusions

In this project, we found that HER2 copy number by fluorescent in-situ hybridization does not correlate with response to trastuzumab containing-therapy. We postulate that other molecular features of the tumor must be relevant for response to trastuzumab in patients selected for trastuzumab treatment based on high level of expression of HER2 or FISH positivity. In addition, serum studies suggest that HER2/ECD is not a useful biomarker in predicting response in trastuzumab-treated patients. Complex interactions between antibody and serum ECD make this marker unreliable as decline is seen in both responding and progressing patients. Finally, circulating tumor cells measured by quantitative RT-PCR after immunomagetic selection show that CK19 is a useful marker for circulating tumor cells, which appear to track with disease burden and response to therapy. HER2 by real-time quantitative PCR lacked sensitivity and specificity necessary for clinical utility.

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List of Personnel Receiving Pay from this Research Effort

Marcia Fournier, Ph.D., Research Fellow
Cinara C. Dias, M.D., M.Sc., Research Fellow

Preoperative Therapy With Trastuzumab and Paclitaxel Followed by Sequential Adjuvant Doxorubicin/Cyclophosphamide for HER2 Overexpressing Stage II or III Breast Cancer: A Pilot Study

By Harold J. Burstein, Lyndsay N. Harris, Rebecca Gelman, Susan C. Lester, Raquel A. Nunes, Carolyn M. Kaelin, Leroy M. Parker, Leif W. Ellisen, Irene Kuter, Michele A. Gadd, Roger L. Christian, Patricia Rae Kennedy, Virginia F. Borges, Craig A. Bunnell, Jerry Younger, Barbara L. Smith, and Eric P. Winer

Purpose: **Trastuzumab combined with chemotherapy improves outcomes for women with human epidermal growth factor receptor 2 (HER2) overexpressing advanced breast cancer. We conducted a pilot study of preoperative trastuzumab and paclitaxel, followed by surgery and adjuvant doxorubicin and cyclophosphamide chemotherapy in earlier stage breast cancer.**

Patients and Methods: **Patients with HER2-positive (2+ or 3+ by immunohistochemistry) stage II or III breast cancer received preoperative trastuzumab (4 mg/kg × 1, then 2 mg/kg/wk × 11) in combination with paclitaxel (175 mg/m² every 3 weeks × 4). Patients received adjuvant doxorubicin and cyclophosphamide chemotherapy following definitive breast surgery. Clinical and pathologic response rates were determined after preoperative therapy. Left ventricular ejection fraction and circulating levels of HER2 extracellular domain were measured serially.**

Results: **Preoperative trastuzumab and paclitaxel achieved clinical response in 75% and complete pathologic response in 18% of the 40 women on study. HER2 3+ tumors were more likely to respond than 2+ tumors (84% v 38%). No unexpected treatment-related noncardiac toxicity was encountered. Four patients developed grade 2 cardiotoxicity (asymptomatic declines in left ventricular ejection fraction). Baseline HER2 extracellular domain was elevated in 24% of patients and declined with preoperative therapy. Immunohistochemical analyses of posttherapy tumor specimens indicated varying patterns of HER2 expression following trastuzumab-based treatment.**

Conclusion: **Preoperative trastuzumab and paclitaxel is active against HER2 overexpressing early-stage breast cancer and may be feasible as part of a sequential treatment program including anthracyclines. The observed changes in cardiac function merit further investigation. Correlative analyses of HER2 status may facilitate understanding of tumor response and resistance to targeted therapy.**

J Clin Oncol 21:46-53. © 2003 by American Society of Clinical Oncology.

PREOPERATIVE SYSTEMIC therapy has been widely used in the treatment of locally advanced and operable breast cancer. Clinical trials of combination chemotherapy as initial treatment for operable breast cancer have consistently demonstrated high rates (> 70%) of clinical response. Complete pathologic response, usually defined as eradication of invasive cancer at the time of histologic analysis, is seen in 10% to 33% of patients receiving preoperative therapy with a variety of different regimens.[1] For instance, preoperative therapy with paclitaxel led to complete pathologic response in 14% of patients with operable breast cancer, comparable to response rates seen with anthracycline-based combination neoadjuvant therapy.[2] Randomized trials have demonstrated that preoperative systemic therapy is as effective as adjuvant chemotherapy with respect to disease-free and overall survival and that it increases the rate of breast-conserving surgery.[1,3] By allowing clinical assessment of tumor response, preoperative therapy constitutes a valuable model for testing new breast cancer treatments.[4,5] Patients with objective tumor response to primary chemotherapy, particularly those with complete pathologic response, have improved long-term cancer outcomes compared with patients who do not respond or have residual invasive tumor.[3,6,7]

Trastuzumab, a high-affinity humanized monoclonal antibody that recognizes the human epidermal growth factor receptor 2 (HER2), is a novel, targeted therapy for breast cancers that overexpress this receptor. Trastuzumab has been evaluated in women with HER2 overexpressing metastatic breast cancer, as a single agent following traditional chemotherapy,[8] as a single agent before chemotherapy,[9] and in combination with a variety of chemotherapy agents. In a large, randomized trial for women with HER2-positive metastatic breast cancer, standard chemotherapy was compared with chemotherapy administered with trastuzumab.[10] Trastuzumab, in combination with either doxorubicin/cyclophosphamide (AC) or paclitaxel, led to higher response rates, longer progression-free survival, and improved overall survival compared with treatment with chemotherapy alone. Thus, trastuzumab combined with chemotherapy has become a standard of care for women with HER2 overexpressing metastatic breast cancer. An unexpected finding in the randomized trial of trastuzumab for metastatic breast cancer was the high rate of clinically significant cardiotoxicity, particularly among patients treated concurrently with trastuzumab and anthracycline-based chemotherapy.[11] The safety and utility of trastuzumab-based therapy for earlier stage breast cancer are not known.

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We developed a preoperative treatment program of trastuzumab in combination with paclitaxel for women with HER2-positive stage II or III breast cancer. Because of available safety data on the use of sequential AC followed by paclitaxel chemotherapy for women with lymph node–positive breast cancer,[12] this regimen became the foundation of the treatment program. To assess the clinical effects of trastuzumab, and because of the demonstrated safety and efficacy of trastuzumab in combination with every-3-week paclitaxel in metastatic disease, we reversed the treatment sequence and administered trastuzumab-paclitaxel before breast surgery. We sought to define the complete pathologic response rate to the trastuzumab/paclitaxel combination and to assess the safety and feasibility of incorporating trastuzumab into a systemic treatment program for early-stage breast cancer that included sequential use of anthracycline-based chemotherapy. In addition, we sought to characterize pathologic changes in response to trastuzumab-based therapy and utility of serologic assays for HER2 in such patients.

PATIENTS AND METHODS

Eligibility

Patients with histologically confirmed invasive breast cancer, clinical stage II or III, including inflammatory breast cancer, were eligible for this study. Patients with clinically negative axillae were required to have primary tumors of > 2 cm on physical examination or mammography. Patients were eligible after diagnostic core needle or incisional biopsy, provided that residual tumor in either the breast or lymph node measured at least 1 cm on mammography, ultrasonography, or physical examination. Patients with bilateral breast cancers were eligible provided that at least one tumor met the clinical staging requirements.

Patients with HER2 2+ or 3+ tumors by immunohistochemistry using a modification of the DAKO HercepTest kit (Dako Corporation, Carpinteria, CA)[13] were eligible. When possible, tumors were reanalyzed for HER2 expression by immunohistochemistry at the time of definitive breast surgery.

Patients with prior history of breast cancer within the previous 2 years, ipsilateral tumor recurrence, prior anthracycline- or taxane-based chemotherapy, or prior high-dose chemotherapy with stem-cell transplant were ineligible.

Patients were required to be more than 18 years of age, with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1, and to be neither pregnant nor nursing. Eligible patients had baseline white blood cells of more than 4,000/mm^3, platelet count more than 100,000/mm^3, bilirubin, and SGOT within institutional limits of normal, and creatinine less than 1.5 mg/dL. Baseline evaluation also included an electrocardiogram to exclude ischemic changes or ventricular hypertrophy and chest radiogram to exclude active cardiac or pulmonary disease. Patients were required to have baseline left ventricular ejection fraction (LVEF) ≥ 50%. Patients with uncontrolled infection, active cardiovascular or pulmonary disease, uncontrolled diabetes, prior malignancy not treated with curative intent, or peripheral neuropathy of any etiology exceeding grade 1 were ineligible for the study. Patients provided written informed consent before enrolling in the study.

This study was conducted in accordance with guidelines established by the United States Department of Health and Human Services. The protocol was reviewed and approved by the institutional review boards of all participating centers. Patients were enrolled between February 1999 and December 2000.

Treatment Plan

Preoperative trastuzumab and paclitaxel. Patients were treated sequentially with preoperative trastuzumab and paclitaxel, followed by definitive breast surgery and then four cycles of AC chemotherapy (Fig 1). Trastuzumab was administered as a one-time loading dose of 4 mg/kg as a 90-minute intravenous infusion, followed by 11 weekly treatments at 2 mg/kg as a 30-minute intravenous infusion, without any planned dose modifications. Starting on the same day as trastuzumab, paclitaxel was administered at 175 mg/m^2 as a 3-hour intravenous infusion every 3 weeks for four cycles. Patients were premedicated with oral dexamethasone 20 mg taken 12 and 6 hours before each treatment and with intravenous administration of diphenhydramine 50 mg and H2-blocker (cimetidine 300 mg, or ranitidine 50 mg) 30 minutes before treatment. Complete blood count and liver function tests were determined on day 1 of each paclitaxel cycle; treatment was permitted if the absolute neutrophil count was > 1,200/mm^3 and platelet count was > 100,000/mm^3. Treatment was deferred 1 week for any toxicity in excess of grade 1. Patients who developed grade 3 nonhematologic toxicity that resolved within 2 weeks were treated with dose reduction of paclitaxel to 135 mg/m^2 for subsequent doses. The protocol permitted G-CSF use as prophylaxis after episodes of febrile neutropenia or for treatment delay of more than 1 week because of neutropenia. Patients who developed grade 3 nonhematologic toxicity that failed to resolve within 2 weeks, or who developed grade 4 nonhematologic toxicity, were taken off study. Patients experiencing anaphylactic hypersensitivity reactions to paclitaxel were taken off study; patients with less severe hypersensitivity reactions were given supportive measures and rechallenged with paclitaxel after additional premedication and at an initially slower rate of paclitaxel infusion. Patients who developed progressive disease (defined below) after two cycles of paclitaxel/trastuzumab neoadjuvant therapy were taken off study.

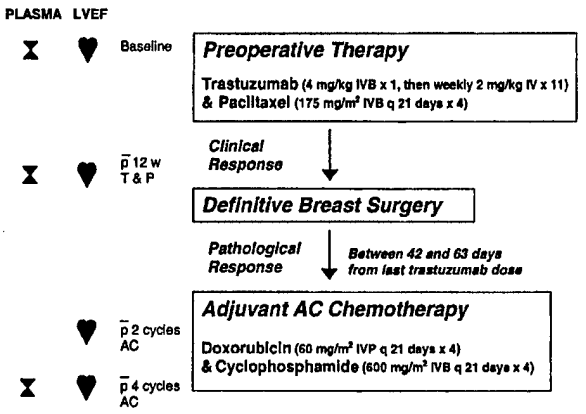

Fig 1. Schema.

Surgery. Definitive breast surgery was performed not less than 7 days and not more than 35 days after the last dose of trastuzumab. Patients underwent either modified radical mastectomy or lumpectomy with complete axillary dissection of the level I and II lymph nodes. Patients with involved or close surgical margins after lumpectomy underwent reexcision or mastectomy to obtain negative margins. To evaluate the feasibility of sentinel lymph node mapping following neoadjuvant therapy, it was suggested that surgeons perform sentinel lymph node mapping at the time of axillary dissection. Mapping was performed according to standard institutional practice using 1 mCi filtered technetium ^{99m}Tc sulfur colloid and 2 to 4 mL 1% isosulfan vital blue dye.

Adjuvant therapy. Adjuvant AC chemotherapy[14] at standard doses and with standard supportive measures began between 2 and 5 weeks after surgery and no less than 6 weeks and no more than 9 weeks after the last trastuzumab dose (Fig 1). This timing was to allow for "washout" of trastuzumab, which has a long half-life.[15] Complete blood count and liver function tests were measured on day 1 of each cycle of adjuvant AC chemotherapy. Patients with toxicity related to AC chemotherapy had treatments delayed until adequate recovery. The protocol permitted G-CSF use as prophylaxis after episodes of febrile neutropenia or for treatment delay > 1 week because of neutropenia.

Patients finished protocol-based therapy at the end of four cycles of adjuvant AC chemotherapy. Patients went on to receive radiation therapy and tamoxifen as indicated by standard practice guidelines.

Cardiac surveillance. LVEF was determined at baseline, after 12 weeks of neoadjuvant trastuzumab/paclitaxel and after cycles 2 and 4 of adjuvant AC chemotherapy (Fig 1). Patients developing symptomatic heart failure, or a decline in ejection fraction of > 20%, were removed from the study.

Measurement of HER2 Extracellular Domain

HER2 extracellular domain (ECD) was measured on plasma samples obtained at baseline, after preoperative therapy and after postoperative therapy, using the Human HER-2 Quantitative ELISA (Oncogene Science, Cambridge, MA) using a sandwich immunoassay according to

manufacturer's guidelines.[16] If the coefficient of variation of duplicates was > 5%, the measurements were repeated.

Study Analysis

Assessment of response. Clinical response was assessed by determining the change in the sum of the products of bidimensionally measurable disease in the breast and ipsilateral axillary lymph nodes as measured on clinical examimation, mammography, ultrasonography, or sectional imaging study (if available). Progressive disease was defined as a 50% increase in sum of the products of bidimensionally measurable disease, or appearance of new lesions elsewhere, after at least two cycles of neoadjuvant therapy. Clinical complete response was defined as disappearance of all clinically detectable cancer in the breast and lymph nodes. Clinical partial response was defined as a decrease of 50% or more in sum of the products of bidimensionally measurable disease in breast and/or lymph nodes. Stable disease referred to all changes in tumor burden not qualifying as progressive disease or clinical complete or partial response. Pathologic complete response (pCR) was defined as complete clinical response with no evidence of microscopic residual invasive tumor in the breast or ipsilateral axillary lymph nodes at the time of definitive breast surgery, based on standard hematoxylin and eosin staining. Patients with residual carcinoma-in-situ but without invasive breast cancer were considered to have pCR. Toxicity was reported using the common toxicity criteria (version 2.0) of the National Cancer Institute.

Statistical methods. Accrual followed a two-stage design with the principal study end point being determination of the pCR rate. In the first phase, 25 patients were to be entered. If 0 or 1 pCRs were observed, accrual would terminate. If two or more pCRs were observed, another 15 patients were to be entered, for a total of 40 patients. It was believed based on historic experience that an observed pCR rate of 15%, representing a total of six or more pCRs among the 40 treated patients, would be of clinical interest and would justify further development of the regimen. If the true pCR rate was only 10%, there was a 27% chance of terminating accrual at the end of the first phase and only a 20% chance of deeming the regimen worthy of further study. If the true pCR rate was 20%, there was a 3% chance of terminating accrual at the end of the first phase and an 83% chance of deeming the regimen worthy of further study.

The 95% confidence interval for pCR was based on the two-stage study design. Response rates were compared using a two-sided Fisher's exact test for the 2 × 2 contingency table.

RESULTS

Patient Characteristics

Forty women with stage II or III, HER2-overexpressing breast cancer participated in the study; median age was 48.5 years (range, 26 to 65 years). Clinical characteristics of the study population are shown in Table 1. One patient was enrolled with an isolated ipsilateral supraclavicular lymph node metastasis as the sole site of stage IV disease. Nearly half the patients had stage III breast cancer, including six patients with inflammatory breast cancer. Half of the patients had clinically detectable axillary lymphadenopathy before start of neoadjuvant treatment. Median preoperative breast tumor size was 5 cm (range 0 to 11 cm). Hormone receptor status and HER2 status as determined by immunohistochemistry are shown in Table 1. Eighty percent of the tumors were HER2 3+ by immunohistochemistry.

Clinical and Pathologic Response to Neoadjuvant Therapy

Clinical and pathologic response rates are shown in Table 2. Complete pathologic response was seen in 18% of all patients (95% confidence interval, 7% to 33%). Residual carcinoma-in-situ was observed in three of the seven women with complete pathologic response and no evidence of residual invasive tumor; two such patients had ductal carcinoma-in-situ, one had lobular carcinoma-in-situ. Patients taken off study for toxicity (n = 1) or who did not proceed to definitive breast surgery after neoadjuvant paclitaxel/trastuzumab (n = 3) were considered to not have a pCR.

Table 1. Baseline Clinical Characteristics of Study Population

	No. Patients	% of Patients
Clinical stage		
II	22	55
IIIA	9	23
IIIB	8	20
T4d (inflammatory)	6	15
IV*	1	2
Clinical nodal status		
Nx/N0	19	47
N1	19	47
N2	2	5
Hormone receptor status		
ER+/PR+	19	47
ER+/PR−	7	18
ER−/PR+	0	
ER−/PR−	14	35
HER2 status		
3+	32	80
2+	8	20
Surgical therapy		
Mastectomy	15	38
Lumpectomy and axillary dissection	21	52
Additional systemic therapy	4	10

*One patient with metastatic disease to the ipsilateral supraclavicular lymph node was included in the study.

Clinical response, either partial or complete, was seen in 75% of patients. The clinical response rate among women with HER2 2+ (n = 8; response rate, 38%) tumors was lower than that seen for women whose tumors were 3+ (n = 32; RR, 84%; $P = .01$). There was no difference in complete pathologic response rates between the HER2 subsets (HER2 3+ pCR in 6 of 32, HER2 2+ pCR in 1 of 8; $P = 1.0$). One patient had evidence of disease progression during treatment. Clinical responses were analyzed separately among patients with measurable tumor in either the breast (n = 39) or lymph node(s) (n = 21; Table 2). The overall clinical response rate did not differ between breast and lymph node sites ($P = .56$).

Response rates were also analyzed as a function of estrogen receptor status and of initial clinical stage (Table 2). Clinical response rates were not significantly different between women with estogen-receptor (ER)-positive or ER-negative tumors ($P = 1.0$). No complete pathologic responses were observed among the six women with inflammatory breast cancer. However, neither clinical response rates ($P = .73$) nor pCR rates ($P = .42$) were significantly different between patients with initial stage II or stage III/IV disease, including inflammatory breast cancer.

Median follow-up was 25 months (range, 9 to 37 months). Seven patients had developed distant metastases. Distant disease-free survival is shown in Fig 2. No recurrences were noted among patients with either complete clinical or complete pathologic response. Four recurrences were noted among the 10 patients without clinical response to neoadjuvant therapy; by contrast, there were three recurrences among the 27 patients with complete or partial clinical response ($P = .052$). Isolated CNS metastases account for three of the seven distant recurrences.

Table 2. Response Rates to Preoperative Trastuzumab and Paclitaxel

	Clinical Response										Pathologic Response	
	N		PD		SD/NA*		cPR		cCR		pCR	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Total	40	100	1	3	9	23	18	45	12	30	7	18†
HER2 status												
3+	32	80	1	3	4	13	16	50	11	34	6	19
2+	8	20	0		5	63	2	25	1	13	1	13
ER status												
Positive	26	65	1	4	6	23	12	46	7	27	4	15
Negative	14	35	0		3	21	6	43	5	36	3	21
Stage												
II	22	55	0		5	23	10	45	7	32	5	23
III/IV	12	30	0		3	25	6	50	3	25	2	17
T4d	6	15	1	17	1	17	2	33	2	33	0	
Clinically measurable tumor site												
Breast	39	98	1	3	9	23	17	44	12	31	8	21‡
LN	21	53	1	5	6	29	2	10	12	57	5	24‡

Abbreviations: PD, progressive disease; SD/NA, stable disease/not assessable; cPR, clinical partial response; cCR, clinical complete response; pCR, pathologic complete response.

*One patient taken off study following paclitaxel hypersensitivity reaction was considered not assessable.

†95% confidence interval 7%–33%.

‡Among patients with clinically measurable disease in breast or lymph node at baseline.

Effect of Preoperative Trastuzumab and Paclitaxel on Tumor HER2 Status

Tumor HER2 status was reanalyzed following preoperative trastuzumab/paclitaxel therapy at the time of definitive surgery. Patients with complete pathologic response (n = 7) had no residual tumor for evaluation. Tumors from six other patients were not assessable because they had stable or progressive disease during preoperative trastuzumab/paclitaxel (n = 4) and did not proceed to surgery or because of inadequate tumor for HER2 testing in the surgical specimen (n = 2). Table 3 shows the HER2 status as measured by immunohistochemistry following preoperative trastuzumab-based therapy. Of the 23 tumors with initial HER2 3+ status available for retesting, 17 retained the 3+ level of expression after trastuzumab and paclitaxel treatment. However, in six cases the HER2 status changed. In four instances, HER2 expression was now scored as 0. In two instances, 2+ expression was reported. Similar trends were seen for tumors originally scored as 2+, although fewer cases were available for analysis.

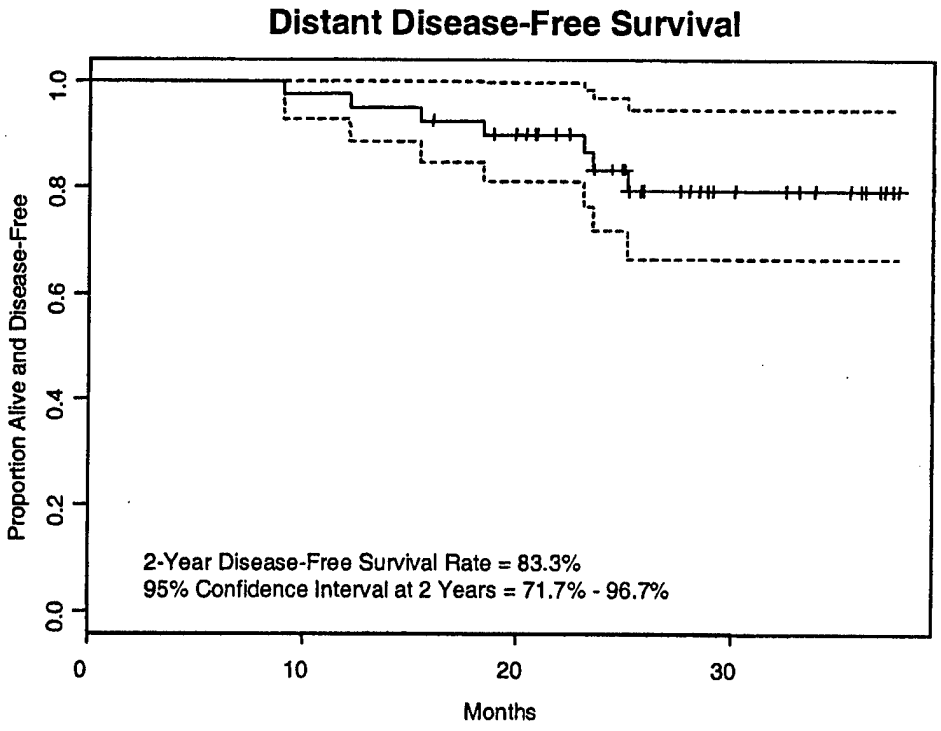

Fig 2. Distant disease-free survival. Kaplan-Meier curve for distant disease-free survival, including confidence intervals.

Table 3. HER2 Status Following Preoperative Trastuzumab/Paclitaxel

	Baseline HER2 Status			
	3+ (n = 32)		2+ (n = 8)	
HER2 Status After Preoperative Therapy	No.	%	No.	%
3+	17	53	1	13
2+	2	6	0	
1+ or 0	4	13	3	37
Not assessable	3	9	3	37
pCR	6	19	1	13

Serologic Assessment of HER2 Extracellular Domain

Serologic measurement of HER2 extracellular domain (ECD) was made at baseline, following preoperative trastuzumab and paclitaxel, and after four cycles of adjuvant AC chemotherapy. Evaluable serum specimens were available for 37 patients at baseline, 30 patients after preoperative therapy, and 22 patients after adjuvant treatment. At baseline, 9 of 37 patients (24%) had serum HER2 ECD levels greater than the cutoff value of 20 ng/mL (mean level, 34.7 ng/mL; range, 21.4 to 57.2 ng/mL). All patients with elevated baseline HER2 ECD had HER2 3+ tumors by immunohistochemistry.

Serial data were available on seven of the nine patients with initially elevated HER2 ECD, and are shown in Fig 3. Serum HER2 ECD levels declined in all seven instances. Serum HER2 ECD levels normalized in the five patients with clinical response during the preoperative trastuzumab and paclitaxel phase of therapy. By contrast, the two patients with either stable or progressive disease had the highest baseline ECD levels, which did not decrease to below the 20 ng/mL value by the end of neoadjuvant therapy.

There were 28 patients with initially negative tests for HER2 ECD. Of these, all remained persistently negative during treatment, with the exception of one patient whose values oscillated around the cutoff value (19.8 → 21.5 → 18.3 ng/mL at baseline, following preoperative treatment, and following adjuvant treatment, respectively).

Treatment Following Preoperative Trastuzumab and Paclitaxel Therapy

After neoadjuvant therapy, most (n = 36) patients went on to definitive breast surgery (Table 1). Twenty-one patients were treated with lumpectomy and axillary node dissection, 15 with mastectomy. Four patients with either stable or progressive disease after preoperative trastuzumab/paclitaxel went on to receive additional systemic therapy before definitive breast surgery; two of these patients subsequently had mastectomy and two lumpectomy. The sentinel lymph node(s) were successfully identified in 11 of these 16 cases in which mapping was attempted. Thirty-four patients received adjuvant AC chemotherapy on study.

Side Effects During Preoperative and Adjuvant Therapy

Side effects encountered during either preoperative or adjuvant therapy are reported in Table 4. In general, the toxicity experience reflected the known side effects of the respective treatments. Paclitaxel-based therapy was commonly associated with anemia, fatigue, hypersensitivity reactions, myalgias, arthralgias, and sensory neuropathy. The adjuvant AC treatment was commonly associated with nausea, vomiting, and fatigue. There was a single instance of febrile neutropenia during adjuvant AC therapy. One patient, included in the study analysis, experienced grade 3 (symptomatic bronchospasm) hypersensitivity reaction with initial paclitaxel administration. Despite extensive premedication, she had recurrent symptoms on rechallenge with paclitaxel and was taken off study.

LVEF was measured at baseline, after 12 weeks of neoadjuvant paclitaxel and trastuzumab, and following cycles 2 and 4 of AC chemotherapy (Table 4; Fig 4). No patients developed symptomatic (grade 3 or 4) heart failure. Changes in LVEF (grade 1: decline of ≥ 10% but < 20%; grade 2: decline of ≥ 20% or below laboratory limit of normal [50% at our institutions]) were defined relative to the baseline LVEF before the start of neoadjuvant treatment. During the neoadjuvant phase of therapy, four patients had grade 1 decline in LVEF, and one patient had a grade 2 decrease. All these patients continued on

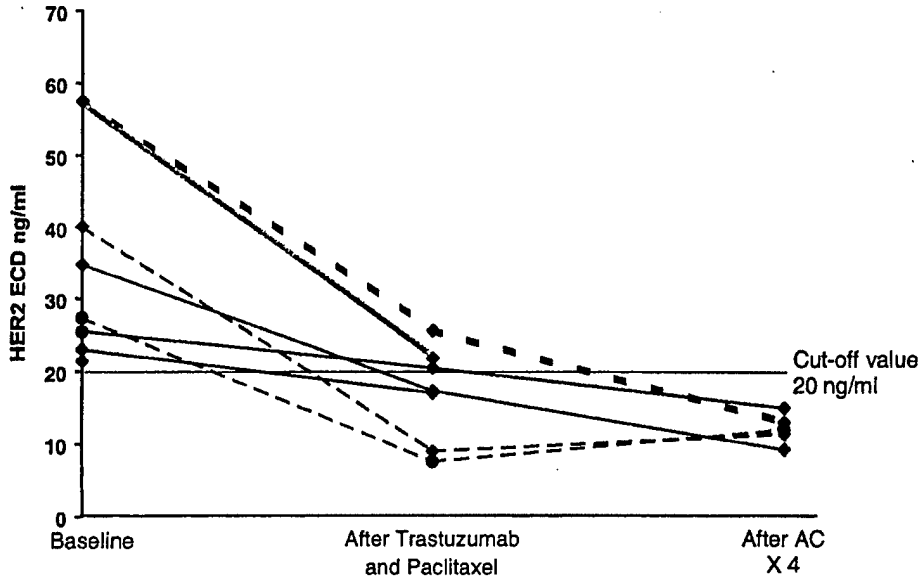

Fig 3. Serial quantitative measurement of HER2 ECD. Patients are identified by response to preoperative trastuzumab and paclitaxel as clinical complete response (thin solid line), clinical partial response (thin dashed line), stable disease (heavy dashed line), or progressive disease (heavy solid line). Serial information was not available on two patients with initially elevated levels.

Table 4. Toxicity Incidence

	Neoadjuvant Paclitaxel and Trastuzumab (n = 40)				Adjuvant Doxorubicin and Cyclophosphamide (n = 34)			
Phase Grade	1	2	3	4	1	2	3	4
Hematologic								
WBC	4	8	2	0	0	7	8	1
ANC	0	3	7	6	0	2	4	6
Hgb	24	0	0	0	1	2	0	0
PLT	0	0	0	0	0	0	0	0
Nonhematologic								
Hypersensitivity Rxn	4	0	1	0	1	0	0	0
Weight gain	2	1	0	0	0	1	0	0
Edema	6	0	0	0	3	0	0	0
Fatigue	13	3	0	0	9	8	1	0
Rash	6	1	0	0	1	1	0	0
Nausea	5	0	1	0	13	10	0	0
Vomiting	1	0	0	0	13	7	0	0
Diarrhea	7	2	0	0	5	1	0	0
Constipation	5	0	0	0	2	3	0	0
Dyspepsia	7	1	0	0	7	1	0	0
SGOT	2	2	0	0	6	0	0	0
SGPT	4	0	0	0	4	0	0	0
Neuropathy—Motor	1	0	0	0	2	0	0	0
Neuropathy—Sensory	18	3	0	0	1	0	0	0
Arthralgia	15	8	1	0	0	0	0	0
Myalgia	22	9	0	0	0	0	0	0
Fatigue	13	3	0	0	9	8	1	0
Hypertension	2	0	1	0	0	0	0	0
LVEF	4	1	0	0	5	4	0	0

with adjuvant therapy. During the adjuvant AC phase of therapy, five patients were observed to have grade 1 toxicity, and four patients had grade 2 toxicity. Newly arising changes in LVEF during AC treatment occurred in three of the five patients with grade 1 toxicity and three of four patients with grade 2 toxicity. The other patients had persistence of changes that appeared during the neoadjuvant paclitaxel/trastuzumab phase of therapy. One patient had LVEF decline from 57% to 40%. She was the only patient to develop an LVEF below 45%, and subsequent determination of LVEF after 3 months showed recovery to 50%.

DISCUSSION

This pilot study sought to examine the safety and efficacy of preoperative therapy with trastuzumab in combination with chemotherapy as part of a multimodality treatment plan for stage II and III breast cancer. This experience with 40 women demonstrated a high rate of clinical activity, with objective responses to trastuzumab in combination with paclitaxel observed in 75% of women. Complete pathologic response was seen in 18% of patients. In general, the noncardiac toxicity experience did not differ from that expected from adjuvant treatment with AC followed by paclitaxel, as reported without the addition of trastuzumab. Sequential therapy, first with trastuzumab and paclitaxel and then with AC, proved feasible. In the available, short follow-up, there have been no recurrences among women whose tumors had complete clinical or pathologic response to preoperative therapy. Anecdotally, a seemingly large percentage of distant recurrences seen to date have been isolated CNS metastases.

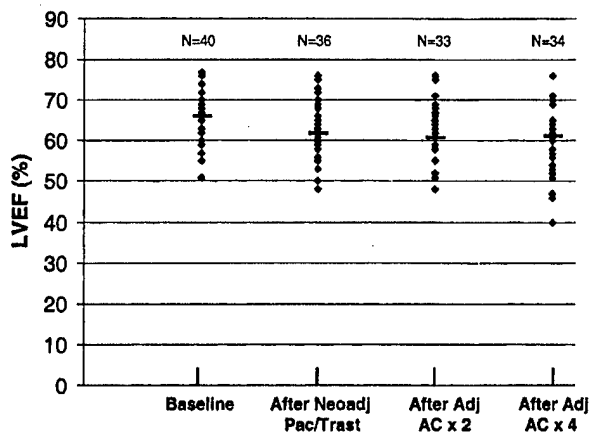

Fig 4. Serial measurement of left ventricular ejection fraction (LVEF). LVEF determinations at various timepoints during protocol treatment. Bars denote median scores.

Pathologic complete response has been shown to predict improved disease-free and overall survival for women treated with anthracycline-based neoadjuvant chemotherapy when compared with women with less than complete pathologic response.[3,7] It remains to be demonstrated whether increased rates of pCR will translate into step-wise improvement in disease-free and overall survival. Previous studies with single-agent paclitaxel as preoperative therapy have yielded complete pathologic response rates of 14%.[2] However, comparing pCR rates between different clinical trials is fraught with difficulty, owing to differences in patient populations, initial tumor burden, duration of therapy, definitions of pathologic clearance, and use of concurrent hormonal treatment. Our a priori hypothesis was that a pCR rate of 15% using trastuzumab-based treatment would be of clinical interest, given the historic rates observed in clinical trials and the relative resistance of HER2-positive tumors to paclitaxel chemotherapy in the metastatic setting.[10] Although the pCR rate to single agent paclitaxel in HER2 positive primary tumors is not known, the observed rate of 18% using trastu-

zumab and paclitaxel is promising, particularly given the relatively advanced stage of presentation in our study population, and justifies further investigation of preoperative trastuzumab-based treatment.

Most of the ongoing randomized adjuvant trials of trastuzumab-based therapy involve treatment with anthracycline-based chemotherapy followed by trastuzumab. Our study shows that up-front therapy with trastuzumab in combination with paclitaxel is possible before standard anthracycline-based chemotherapy. There is reason to believe that earlier initiation of trastuzumab-based treatment with chemotherapy may be superior to later use of trastuzumab-based treatment. When used as monotherapy among women with advanced breast cancer, trastuzumab achieved higher response rates as first-line treatment than when administered after tumor progression on chemotherapy.[8,9] The schema in our study potentially lends itself to use in trials addressing the sequencing of trastuzumab and chemotherapy for early-stage breast cancer.

Cardiac toxicity is an ongoing concern related to use of trastuzumab, particularly for women with more favorable long-term prognoses. In our study, trastuzumab was not administered concurrently with anthracycline-based chemotherapy, and a planned delay was introduced to minimize overlap between trastuzumab and initiation of anthracycline treatment. No patients developed symptomatic congestive heart failure. A number of patients did develop asymptomatic declines in their ejection fraction, and four patients dropped their ejection fraction to a level below institutional limits of normal (50%)—three during the AC phase of therapy. At the time the study was initiated, the half-life of trastuzumab was believed to be on the order of 7 days. Subsequent data indicate a half-life of 28 days.[15] Thus, our attempts to reduce overlap between trastuzumab and anthracycline exposure may have been less effective than anticipated, and this overlap may account for the changes in left ventricular function that were observed in the trial. Recently, the ECOG reported preliminary cardiac toxicity data from a pilot study of sequential adjuvant treatment with trastuzumab-paclitaxel, followed by AC, followed by maintenance trastuzumab therapy.[17] In the ECOG trial, median LVEF declined from 63% to 59% through the AC phase of therapy, with 6% of patients developing an LVEF less than normal after AC treatment. Collectively, our study and the ECOG results provide some reassurance that sequential trastuzumab-paclitaxel followed by AC chemotherapy is not likely to be associated with prohibitive short-term cardiotoxicity. However, experience from randomized trials of adjuvant trastuzumab will be needed to define the short- and long-term cardiac sequelae.

This clinical trial included patients whose tumors were either HER2 3+ or 2+ by immunohistochemistry, based on treatment standards for women with metastatic breast cancer at the time of study accrual. Data on HER2 gene amplification were not collected prospectively. The rapidly evolving literature on HER2 testing indicating that the vast majority of patients whose tumors are 3+ will, in fact, be fluorescence in situ hybridization positive; by contrast, most 2+ tumors will not have HER2 gene amplification. It is likely that only those patients with tumors that are 3+ and/or fluorescence in situ hybridization-positive derive substantial clinical benefit from trastuzumab-based therapy.[18] Thus, the clinical activity for trastuzumab-based therapy reported in this trial may differ modestly from that seen among patients selected using different pathologic criteria.

The HER2 status of breast tumors treated with trastuzumab has not previously been reported. In this study, we analyzed HER2 expression before and after trastuzumab and paclitaxel therapy. For most patients with residual tumor after 12 weeks of neoadjuvant treatment, HER2 expression as measured by immunohistochemistry was unchanged. However, a subset of patients whose initial tumors were 3+ were found, on testing after induction therapy, to have lost immunohistochemical expression of HER2. The clinical significance of this finding is not known. It may represent downregulation of HER2 expression following anti-HER2 antibody exposure, as reported in preclinical tumor models.[19] It may also represent intrinsic heterogeneity of HER2 expression and tumor response, or an artifact of tumor sampling or testing. It is not clear whether this finding implies resistance or sensitivity to trastuzumab. Further studies of tumor changes at the cellular and molecular level brought on by trastuzumab therapy are warranted.

The role of HER2 ECD in selecting patients for trastuzumab-based therapy, or monitoring response to such therapy, remains unclear.[20] A recent report indicates that changes in HER2 ECD correlate with response to trastuzumab-taxane therapy in women with HER2-positive metastatic breast cancer.[21] Our results indicate similar findings among patients with stage II/III breast cancer, but larger prospective trials will be needed to define whether HER2 ECD should be routinely and/or serially measured.

The administration of trastuzumab for patients with early-stage breast cancer remains investigational. Our trial of preoperative therapy demonstrates the feasibility of using trastuzumab treatment as part of a multimodality treatment program for stage II and III breast cancer. Other studies using trastuzumab in combination with different antineoplastic agents, and in other sequences of treatment, may further define possible treatment approaches that incorporate trastuzumab-based therapy into early-stage breast cancer, while we await the results from large, randomized studies.

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Trastuzumab and Vinorelbine as First-Line Therapy for HER2-Overexpressing Metastatic Breast Cancer: Multicenter Phase II Trial With Clinical Outcomes, Analysis of Serum Tumor Markers as Predictive Factors, and Cardiac Surveillance Algorithm

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Purpose: **Trastuzumab-based therapy improves survival for women with human epidermal growth factor receptor 2 (HER2)–positive advanced breast cancer. We conducted a multicenter phase II study to evaluate the efficacy and safety of trastuzumab combined with vinorelbine, and to assess cardiac surveillance algorithms and tumor markers as prognostic tools.**

Patients and Methods: **Patients with HER2-positive (immunohistochemistry [IHC] 3+-positive or fluorescence in situ hybridization [FISH]-positive) metastatic breast cancer received first-line chemotherapy with trastuzumab and vinorelbine to determine response rate. Eligibility criteria were measurable disease and baseline ejection fraction ≥ 50%. Serial testing for HER2 extracellular domain (ECD) was performed.**

Results: **Fifty-four women from 17 participating centers were entered onto the study. The overall response rate was 68% (95% confidence interval, 54% to 80%). Response rates were not affected by method of HER2 status determination (FISH v IHC) or by prior adjuvant chemotherapy. Median time to treatment failure was 5.6 months; 38% of patients were progression free after 1 year. Concurrent therapy was quite feasible with maintained dose-intensity. Patients received both chemotherapy and trastuzumab on 90% of scheduled treatment dates. Two patients experienced cardiotoxicity in excess of grade 1; one patient experienced symptomatic heart failure. A surveillance algorithm of screening left ventricular ejection fraction (LVEF) at 16 weeks successfully identified women at risk for experiencing cardiotoxicity. Other acute and chronic side effects were tolerable. Lack of decline in HER2 ECD during cycle 1 predicted tumor progression.**

Conclusion: **Trastuzumab and vinorelbine constitute effective and well-tolerated first-line treatment for HER2-positive metastatic breast cancer. Patients with normal LVEF can be observed with surveillance of LVEF at 16 weeks to identify those at risk for cardiotoxicity.**

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THERAPY FOR metastatic breast cancer is guided by biologic features of the tumor. Women with hormone-receptor–positive tumors are candidates for endocrine therapies, and women with human epidermal growth factor receptor 2 (HER2)–overexpressing tumors are candidates for trastuzumab, a humanized monoclonal antibody directed against the 185-kd HER2 protein.[1] Compared with chemotherapy alone, chemotherapy with trastuzumab improves clinical outcomes, including response rate, time to progression, and overall survival for women with HER2-positive, metastatic breast cancer.[2] However, trastuzumab therapy can be associated with cardiotoxicity. Subsequent retrospective analyses have indicated that concurrent exposure to anthracyclines and advanced age are predictors of trastuzumab-related cardiac dysfunction.[3]

Because of the improvement in survival, trastuzumab-based therapy has become a standard of care for women with HER2-positive advanced breast cancer. However, neither the optimal trastuzumab-based regimen nor the optimal duration of therapy with trastuzumab has been characterized. Preclinical data have indicated favorable interactions between trastuzumab and a variety of chemotherapeutic agents.[4] Certain alkylators, taxanes, and combinations of taxanes and platinum salts, as well as vinorelbine, have reproducibly exhibited synergistic interactions with trastuzumab in laboratory analyses of growth of HER2-overexpressing breast tumor cell lines. For that reason, and because vinorelbine therapy is not associated with cardiotoxicity, alopecia, or significant gastrointestinal side effects, an initial phase II study of trastuzumab and vinorelbine was performed.[5] In that single-center study, combination therapy with trastuzumab and vinorelbine yielded objective response in 75% of

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patients, including robust response rates in women with previous anthracycline and taxane treatment. This compared favorably to historic response rates with vinorelbine therapy, which have averaged between 16% and 40% for similar patients,[6] and to monotherapy with trastuzumab, which has yielded response rates of 15% to 30%.[7] There was no clinically apparent cardiotoxicity; two patients had asymptomatic declines in ejection fraction. Treatment with the combination regimen was delivered more than 93% of the time—an important consideration when trying to capture synergistic interactions between two agents.

The initial study of trastuzumab and vinorelbine was a relatively small, single-institution trial; it included patients with extensive prior therapy, as well as patients with tumors that were HER2 2+-positive and 3+-positive as determined by immunohistochemistry (IHC). Because of evolving changes in treatment, we sought to characterize the clinical value of such a regimen as first-line treatment, particularly among women with tumors that were strongly HER2-positive (ie, either 3+-positive by IHC or having evidence of gene amplification when measured by fluorescence in situ hybridization [FISH]); retrospective analyses have indicated that such patients are most likely to benefit from trastuzumab-based treatment.[8] We sought to extend the safety and efficacy experience in a multicenter trial to more accurately gauge the value of the regimen and to assess a practical algorithm for cardiac function surveillance in women receiving trastuzumab-based therapy. In addition, we wanted to evaluate the utility of serologic testing for HER2 extracellular domain (HER2 ECD) in the serum of patients receiving trastuzumab-based therapy. HER2 ECD is a prognostic factor that has been extensively evaluated among patients with advanced breast cancer but not in patients receiving trastuzumab.[9] A multicenter, phase II study of trastuzumab and vinorelbine was developed to examine these clinical questions.

PATIENTS AND METHODS

Eligibility

Eligible patients had stage IV breast cancer, with measurable disease according to the Response Evaluation Criteria in Solid Tumors Group criteria, and no active CNS metastases. Patients with asymptomatic brain metastases were eligible if they had finished local therapy more than 3 months before enrollment. Patients were required to be 18 years of age or older, with Eastern Cooperative Oncology Group performance status 0 to 2 and life expectancy greater than 3 months. Patients could not have received prior chemotherapy for metastatic breast cancer or prior vinorelbine. Prior adjuvant treatment with trastuzumab was permitted for patients who were 1 year from the conclusion of that therapy. Adjuvant chemotherapy was permitted, provided that the cumulative doxorubicin dosage did not exceed 360 mg/m^2 and that patients were at least 3 weeks from the conclusion of treatments. Prior hormone therapy for early-stage or metastatic breast cancer was permitted. Patients had to have concluded prior radiation therapy at least 14 days before enrollment. Any previous hormonal or other biologic therapy was discontinued at the time of study entry.

Eligibility criteria were absolute neutrophil count more than 1,500/μL, platelet count more than 100,000/μL, bilirubin less than 2 mg/dL, AST $\leq$ 3 times upper limit of normal, glucose less than 200 mg/dL, and left ventricular ejection fraction (LVEF) $\geq$ 50% as determined by echocardiogram or radionuclide ventriculography. Patients with pre-existing neuropathy in excess of grade 1 were ineligible, as were patients with serious illness or a medical or psychiatric condition that might interfere with their ability to provide informed consent or receive protocol-based therapy.

Table 1. Vinorelbine Dose Modification for Blood Counts on Day of Treatment

Absolute Neutrophil Count (μL)	Platelet Count (μL)	Dose Adjustment	Vinorelbine Dose (mg/m^2)
> 1,250	> 100,000	No change	25
750-1,250	50,000-99,000	Decrease 40%	15
< 750	< 50,000	Delay 1 week	

NOTE. Patients requiring treatment delay > 3 weeks for hematologic toxicity were taken off study.

Patients were required to have tumors with documented overexpression of HER2, either 3+-positive by IHC or with gene amplification by FISH, according to routine pathology and laboratory methods at each participating center.

All patients provided written informed consent before entering onto the study. The protocol was reviewed and approved by the institutional review boards at all participating centers. The study was conducted in accordance with guidelines established by the United States Department of Health and Human Services. Patients were entered onto the study between October 2000 and October 2001.

Treatment Plan

The initial trastuzumab infusion was 4 mg/kg intravenously (IV), administered over the course of 90 minutes. Patients were observed for 60 minutes after the first infusion. Subsequently, trastuzumab was given weekly at 2 mg/kg IV, administered over the course of 30 minutes. There was no dose modification for trastuzumab. If patients experienced an infusion syndrome characterized by rigors, fever, or other symptoms of hypersensitivity to trastuzumab, treatment was stopped, and patients were assessed and given supportive measures (acetaminophen, diphenhydramine, H2 antagonists, dexamethasone, or meperidine) as needed. Treatment was reinstituted when vital signs were stable. Trastuzumab was given as a weekly 90-minute infusion until the treatment was tolerated without adverse effects. Trastuzumab was administered on days when vinorelbine was not administered (see next paragraph).

Vinorelbine was given weekly on the same days as trastuzumab, after trastuzumab administration. The dose of vinorelbine was 25 mg/m^2, administered through a free-flowing IV line as a 6- to 10-minute IV infusion, followed by 125 mL of saline solution. Patients were assessed with weekly complete blood count with differential, and liver function tests every 4 weeks. The protocol called for the vinorelbine dose to be adjusted each week on the day of therapy, on the basis of hematologic toxicity, as shown in Table 1. The protocol permitted granulocyte colony-stimulating factor use for treatment delay of more than 2 weeks that resulted from neutropenia or febrile neutropenia. The vinorelbine dose was reduced to 12.5 mg/m^2 if the bilirubin level was between 2 and 3 mg/dL and was not administered if the bilirubin level was more than 3 mg/dL. Patients who developed grade 2 neurologic toxicity were to receive vinorelbine at 15 mg/m^2 until toxicity resolved to grade 1 or lower. Patients with treatment-related grade 3 nonhematologic toxicity did not receive vinorelbine therapy until the toxicity resolved to grade 1 or lower. If toxicity failed to resolve with a 2-week treatment delay, patients were taken off protocol. Patients with treatment-related grade 4 nonhematologic toxicity were taken off study.

Patients had LVEF measured at baseline and at week 16 on study (ie, after two cycles of therapy). Patients experiencing absolute declines in LVEF of greater than 20% had cardiac surveillance with LVF determination every 8 weeks. Patients were taken off study if they experienced grade 3 cardiac toxicity (symptomatic congestive heart failure) or if they developed LVEF less than 40%. Those patients with no cardiac symptoms, LVEF $\geq$ 40% at week 16, and no LVEF decline of more than 20% were not required to have ongoing surveillance. Investigators could re-evaluate LVEF as clinical circumstances warranted.

HER2 ECD

Serum testing for HER2 ECD was done at baseline; at weeks 2, 3, 5, and 8 of cycle 1; and at the end of every subsequent treatment cycle. HER2 ECD

levels were determined with a murine sandwich enzyme immunoassay according to the manufacturer's instructions (Oncogene Science, Inc, Cambridge, MA).[10] The predictive utility of HER2 ECD was evaluated using receiver operating characteristic (ROC) curve analysis with P values less than .05 considered significant.[11]

Study Analysis

Patients received weekly therapy and were restaged every 8 weeks. Tumor response was defined according to the Response Evaluation Criteria in Solid Tumors Group criteria.[12] Those patients with stable disease or with either complete response (CR) or partial response (PR) received ongoing treatment with 8-week cycles of therapy, followed by restaging. Patients with progressive disease were taken off study. Toxicity was recorded according to National Cancer Institute common toxicity criteria (version 2.0).

The primary end point of this study was overall response (CR% plus PR%) on the basis of all eligible patients who received at least one dose of protocol therapy. The accrual goal was 52 eligible patients; a total of 55 patients were entered onto the study, to ensure that 52 patients would be eligible. The study design was based on the assumption that if at least 27 of these patients (52%) were to have a response, the combination of trastuzumab and vinorelbine would be deemed useful for additional development in phase III trials; if 26 or fewer (≤ 50%) were to have a response, the regimen would not proceed to phase III development. If the true response rate of HER2-positive patients to this regimen were 40% (ie, close to the response rate of vinorelbine alone as first-line therapy), there would be only a 5% chance of proceeding to a phase III study. If the true response rate were 60%, there would be a 91% chance of proceeding to a phase III study. With 55 total patients, the 95% confidence interval (CI) for any particular toxicity would be no wider than 28%.

All patients who met inclusion criteria and exclusion criteria and enrolled onto the study were included in the analysis, to determine response rate. Eligible patients removed from treatment because of toxicity, withdrawal of consent, or death as a result of any cause were counted in the denominator for computing the proportion of responses.

RESULTS

Clinical Efficacy

A total of 55 patients from 17 participating centers in the United States entered onto the study. One patient was ineligible, did not receive protocol-based therapy, and was not included in the study analysis; the data analyses reflect the experience of the other 54 patients. The demographic characteristics of the study patients are shown in Table 2. Patients had a median of three sites of tumor burden; the vast majority of patients (81%) had visceral sites (eg, lung, liver) of metastatic disease. Eighty percent of patients were eligible by virtue of HER2 status determined by IHC; 20% were eligible by virtue of by FISH. Roughly half of the patients had tumors that were estrogen receptor (ER)-positive and HER2-positive, and nearly two thirds had received adjuvant chemotherapy.

Objective response, either CR or PR, was seen in 37 of 54 patients (overall response rate 68%; 95% CI, 54% to 80%; Table 3). Four patients had CRs, 33 had PRs, and an additional nine patients had stable disease for 6 months or longer. Response rates were analyzed among clinical subsets of patients, defined by HER2 status, hormone-receptor status, and prior adjuvant chemotherapy treatment (Table 4). There were no statistically significant differences in response rate between patients who entered onto the study with IHC 3+-positive or FISH-positive tumors, or between hormone-receptor–positive tumors versus hormone-receptor–negative tumors, or between patients who had received adjuvant chemotherapy and those who had not.

At the time of study analysis, four patients remained on treatment, and 50 patients had come off study. Of those 50 patients, 31 had experienced tumor progression, including five who had isolated

Table 2. Patient Characteristics

	No. of Patients	%
Age, years		
Median	54.5	
Range	29-82	
No. of sites of disease		
Median	3	
Range	1-6	
ECOG performance status		
0	38	70
1	15	28
2	1	2
Sites of disease		
Lung or pleura	29	54
Liver	28	52
Lymph node	28	52
Bone	25	46
Breast or chest wall	18	33
Soft tissue	12	22
Skin	4	7
HER2 status		
IHC 3+-positive	43	80
FISH-positive	10	18
Other*	1	2
Hormone receptor status		
ER-positive PR-positive	20	37
ER-positive PR-negative	6	11
ER-negative PR-positive	4	7
ER-negative PR-negative	24	44
Adjuvant chemotherapy		
No	20	37
Yes	34	63
Adjuvant regimens		
CMF	5	9
Anthracycline based	19	35
Taxane based	1	2
Anthracycline and taxane based	9	17

Abbreviations: ECOG, Eastern Cooperative Oncology Group; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; ER, estrogen receptor; PR, progesterone receptor; CMF, cyclophosphamide, methotrexate, fluorouracil.

*One center reported IHC results as "strongly positive membranous staining" without a quantified score; this patient was considered IHC 3+-positive in subsequent analyses.

Table 3. Overall Response Rates

Best Response	No. of Patients	Response Rate (%)
Total	54	
CR	4	7%
PR	33	61%
CR + PR	37	68%*
Stable disease ≥ 6 months	9	17%
Progressive disease	8	15%

Abbreviations: CR, complete response; PR, partial response.
*95% confidence interval, 54-80%.

Table 4. Response Rates Among Clinical Subsets of Patients

	No. of Patients	No. of Responders	Response Rate (%)	Fisher Exact *P*
HER2 status				
IHC 3+-positive	44	30	68	.99
FISH-positive	10	7	70	(IHC *v* FISH)
Hormone receptor status				
ER-positive PR-positive	20	16	80	.075
ER-positive PR-negative	6	4	67	(HR-positive *v* HR-negative)
ER-negative PR-positive	4	4	100	
ER-negative PR-negative	24	13	54	
Adjuvant chemotherapy				
No	20	14	70	.99
Yes	34	23	68	
Adjuvant regimens				
CMF	5	5	100	.14
Anthracycline based	19	12	63	(anthracycline *v* nonanthracycline)
Taxane based	1	1	100	
Anthracycline and taxane based	9	5	56	

Abbreviations: HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; ER, estrogen receptor; PR, progesterone receptor; CMF, cyclophosphamide, methotrexate, fluorouracil; HR, hormone receptor.

progression in the CNS. Among the remaining 19 patients withdrawn from study before overt tumor progression, seven patients withdrew after achieving optimal chemotherapy response and continued receiving trastuzumab monotherapy. An additional seven patients withdrew consent for physician or patient preference. Five patients went off study for excessive toxicity. Figure 1 shows the time to treatment failure; patients electing to go off study for personal preference or optimal tumor response are censored. Patients were on study for a median of 5.6 months (range, 0.46 to 16+ months). A substantial fraction of patients (38%) remained free of progression through 12 months of treatment.

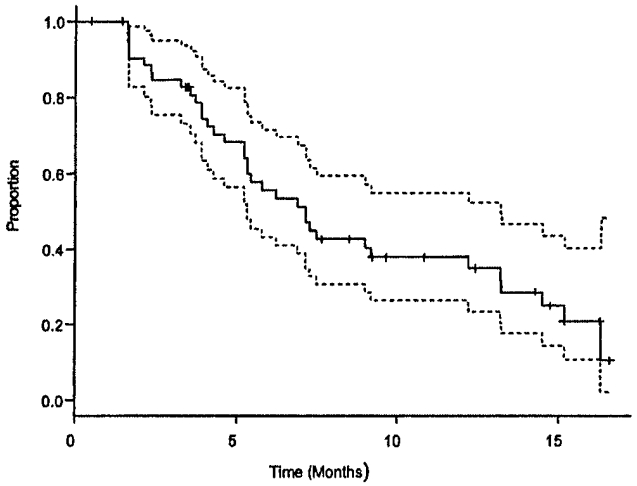

Fig 1. Time to treatment failure. Proportion of patients without treatment failure caused by toxicity or progressive disease. Patients coming off study for optimal tumor response or personal preference are censored. (- - - -) show 95% confidence intervals.

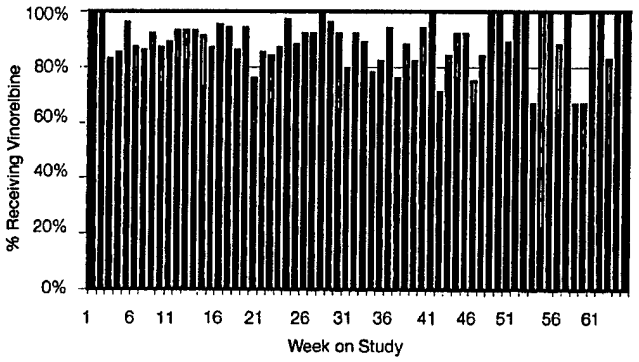

Fig 2. Percentage of patients receiving weekly vinorelbine chemotherapy.Percentage of patients receiving vinorelbine therapy at full dose (darkened portion of bar) or reduced dose (open portion of bar) for each week of study analysis, for patients remaining on study.

Dosing and Toxicity

Protocol therapy called for ongoing weekly trastuzumab treatment without dose modification and administration regardless of vinorelbine dosing on days of treatment. In contrast, vinorelbine dose in this study was adjusted week to week, on the basis of laboratory parameters, as shown in Table 1. A total of 1,634 treatment weeks were recorded on study. Vinorelbine was given at full dose (25 mg/m^2) in 1,226 (75%) treatment weeks, and a reduced dose (15 mg/m^2) was given in 243 (14.9%) treatment weeks. Thus, in 90% of the treatment weeks, concurrent administration of chemotherapy and trastuzumab could be accomplished. Vinorelbine was omitted in 165 treatment weeks (10%) for the following reasons: neutropenia, 67 weeks (4%); patient-scheduling arrangements (eg, travel), 55 weeks (3.4%), coordination of surgical procedures (eg, installation of indwelling catheters), 10 weeks (0.6%); minor upper respiratory or cardiovascular symptoms, 7 weeks (0.4%); patient preference, 11 weeks(0.7%); other reasons, 15 weeks (0.9%).

To assess the effect of cumulative and prolonged chemotherapy treatment, we analyzed the administration of chemotherapy for each week over time. Figure 2 shows the percentage of patients receiving vinorelbine chemotherapy—either full dose or reduced dose—for each week on study. Despite prolonged courses of treatment in some patients, most women continued to receive weekly chemotherapy. Figure 3 shows the relative dose-intensity of vinorelbine chemotherapy over time, normal-

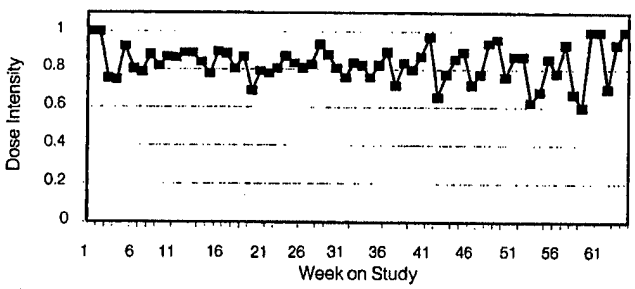

Fig 3. Vinorelbine relative dose-intensity normalized to 1 = 25 mg/m^2, for each week of protocol treatment among patients remaining on study.

Table 5. Frequency of Treatment-Related Toxicity

Type	Total		NCI Common Toxicity Criteria							
			Grade 1		Grade 2		Grade 3		Grade 4	
	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Hematologic										
Leukocytes	30	56	3	6	12	22	15	28	0	0
Neutrophils	39	72	1	2	8	15	21	39	9	17
Hemoglobin	35	65	16	30	16	30	2	4	1	2
Platelets	2	4	2	4	0	0	0	0	0	0
Febrile neutropenia	2	4	0	0	0	0	2	4	0	0
Nonhematologic										
Allergic rhinitis	12	22	8	15	4	7	0	0	0	0
Fever without neutropenia	17	31	12	22	5	9	0	0	0	0
Fatigue	43	80	24	44	15	28	4	7	0	0
Rigors/chills	10	19	9	17	1	2	0	0	0	0
Weight loss	6	11	4	7	2	4	0	0	0	0
Alopecia	17	31	13	24	4	7	0	0	0	0
Injection site reaction	9	17	7	13	2	4	0	0	0	0
Rash/desquamation	7	13	6	11	1	2	0	0	0	0
Hot flashes	8	15	5	9	3	6	0	0	0	0
Anorexia	15	28	13	24	2	4	0	0	0	0
Constipation	21	39	16	30	5	9	0	0	0	0
Dyspepsia	7	13	4	7	3	6	0	0	0	0
Nausea	29	54	24	44	5	9	0	0	0	0
Vomiting	14	26	10	19	4	7	0	0	0	0
Stomatitis	16	30	12	22	4	7	0	0	0	0
Diarrhea	19	35	17	31	0	0	1	2	1	2
Alkaline phosphatase	6	11	6	11	0	0	0	0	0	0
AST or ALT	10	19	8	15	2	4	0	0	0	0
Infection—other	13	24	7	13	5	9	1	2	0	0
Hyperglycemia	8	15	5	9	2	4	1	2	0	0
Arthralgia	10	19	5	9	5	9	0	0	0	0
Myalgia	15	28	12	22	2	4	1	2	0	0
Headache	15	28	11	20	4	7	0	0	0	0
Abdominal pain or cramping	11	20	7	13	2	4	2	4	0	0
Dizziness or lightheadedness	7	13	7	13	0	0	0	0	0	0
Insomnia	15	28	14	26	1	2	0	0	0	0
Mood alteration or depression	9	17	6	11	3	6	0	0	0	0
Neuropathy, sensory	24	44	18	33	6	11	0	0	0	0
Thrombosis or embolism	3	6	0	0	0	0	3	6	0	0
Cardiac, LVEF	8	15	6	11	1	2	1	2	0	0

Abbreviations: NCI, National Cancer Institute; LVEF, left ventricular ejection fraction.

ized at 1 for patients receiving 25 mg/m^2. The weekly relative dose-intensity was well preserved between 0.8 and 1.0 through more than 1 year of therapy (median, 0.83).

The incidence and severity of acute toxicity associated with combined trastuzumab-vinorelbine therapy was quite low. All treatment-related toxicities that occurred in more than 10% of patients on study are shown in Table 5. There were two patients with febrile neutropenia; both recovered without sequelae. The nonhematologic toxicity profile shown was also quite favorable, with few grade 3 or 4 toxicity events. One patient had grade 3 cardiotoxicity (see *Cardiac Surveillance and Cardiotoxicity*). Three patients had thromboembolic events; two patients had upper extremity deep-vein thromboses associated with indwelling venous catheters, and one patient had a pulmonary embolism. Modest degrees of fatigue, injection-site reactions, and peripheral neuropathy were observed, which were not treatment limiting. Fewer than one third of patients had any degree of alopecia, and gastrointestinal symptoms were also mild, with less than 10% of patients having grade 2 constipation and no patients having grade 3 or 4 gastrointestinal toxicities. Side effects related to chronic chemotherapy exposure, such as fluid retention, nail changes, phlebitis, or hand-foot syndrome, were rarely encountered, if at all.

Cardiac Surveillance and Cardiotoxicity

Eligible patients began the study with LVEF 50% or greater and were screened with a single reanalysis of LVEF at week 16 on study. One patient developed grade 3 cardiotoxicity during the second 8-week cycle of therapy, with symptoms of heart failure and LVEF of 41%, decreased from 62% at baseline. Her symptoms resolved with appropriate medical therapy. A second patient had asymptomatic grade 2 cardiotoxicity with LVEF that declined to 40% after 7 months on study (Table 5).

The median LVEF at baseline was 64% (Fig 4). At week 16, 44 of 54 patients were rescreened for LVEF; median LVEF was

unchanged at 64%. The other 10 patients either were off study or declined to be retested. The cardiac outcomes as a function of the week 16 surveillance point are noted in Figure 4. None of the 42 patients with week 1 LVEF greater than 50% experienced cardiotoxicity in excess of grade 1. In contrast, of the two patients noted at week 16 to have LVEF 50% or less, one developed grade 2 and one developed grade 3 cardiotoxicity with protocol-based therapy.

Serum Testing of HER2 ECD

Levels of HER2 ECD were determined at baseline and serially throughout treatment. Levels at baseline and at the end of cycle 1 were available for 43 patients. Figure 5A shows the absolute change in serum HER2 ECD levels through one cycle of trastuzumab-vinorelbine therapy. Most patients had a decline in serum levels, including patients with clinical response (either CR or PR; blue lines) or stable disease (green line) at the end of cycle 1. We performed exploratory analyses of the predictive value of HER2 ECD, and examined both baseline levels and changes with one cycle of treatment. Neither the baseline level of HER2 ECD nor a decrease in HER2 ECD with therapy predicted clinical response after one cycle of treatment. However, a lack of decline in HER2 ECD was a predictor for tumor progression after cycle 1, as shown in the ROC curve (Fig 5B).

DISCUSSION

This multicenter phase II trial of combination therapy with trastuzumab and vinorelbine as first-line treatment for HER2-positive metastatic breast cancer demonstrated high rates of clinical activity achieved with limited acute toxicity. More than two thirds of patients had objective response, and nearly 40% of patients were without disease progression at 1 year. Prior adjuvant chemotherapy did not affect response rates. Because laboratory data indicate synergy between vinorelbine and trastuzumab, it was important to assess the long-term feasibility of concurrent treatment. Patients received combined therapy in 90% of treatment weeks, and dose-intensity was well maintained through extended periods of treatment. Cardiac toxicity, a side effect of particular concern in trastuzumab treatment, was acceptably low; only one patient developed symptomatic heart failure. A cardiac-screening algorithm was used that reassessed

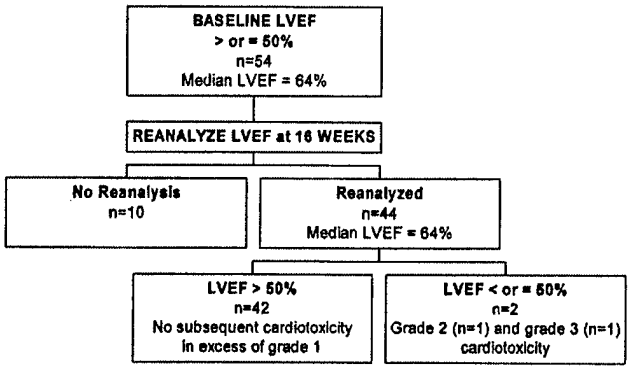

Fig 4. Cardiac surveillance strategy and outcomes. LVEF, left ventricular ejection fraction.

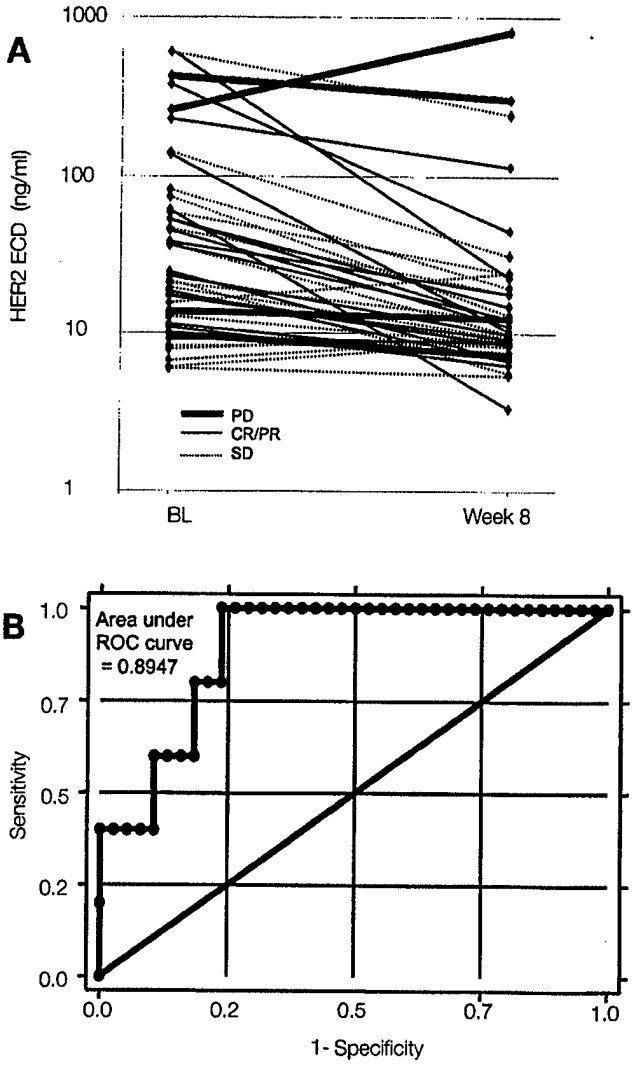

Fig 5. Serum human epidermal growth factor receptor 2 (HER2) extracellular domain (ECD) in patients treated with trastuzumab and vinorelbine. (A) Change in HER2 ECD during cycle 1 of therapy; (B) Receiver operating characteristic for progressive disease (PD) with change in baseline (BL) HER2 ECD during cycle 1. CR, complete response; PR, partial response.

LVEF after 16 weeks of therapy. Among patients receiving ongoing protocol-based treatment, the finding of LVEF $\leq$ 50% at 16 weeks appeared to identify patients at risk for grade 2 or greater cardiotoxicity. Among patients with LVEF greater than 50%, subsequent grade 2 or greater cardiotoxicity was not encountered. There was a low incidence of peripheral neuropathy, perhaps in part because patients had not previously received neurotoxic chemotherapy for advanced breast cancer.

Other phase II trials have demonstrated response rates on the order of 60% to 80% for combinations of trastuzumab with taxanes,[13-15] vinorelbine,[5,16] or triplets of trastuzumab with taxanes and platinum salts.[17] Preliminary analysis of a randomized trial of trastuzumab with paclitaxel versus trastuzumab in combination with paclitaxel and carboplatin indicates improvement in time to progression with the triplet combination.[18] Other trials that have

compared polychemotherapy to monotherapy for metastatic breast cancer have frequently shown improvements in progression-free survival, but not overall survival, with the use of multiagent regimens.[19]

Comparisons among these several trials are fraught with difficulty, owing to different definitions of HER2 overexpression and varying degrees of prior therapy. Given these limitations, the results of treatment with trastuzumab and vinorelbine are generally comparable in terms of response rate and time to progression with other reports of single-agent chemotherapy with trastuzumab. Almost 40% of patients had disease that was either responsive or stable through 1-year of treatment. A substantial fraction of patients elected to end study treatment before experiencing disease progression in favor of trastuzumab monotherapy or trastuzumab with hormonal therapy; this result is a testimony to the palliative efficacy of the regimen. However, neither the clinical benefit of ongoing trastuzumab therapy in such circumstances nor the impact of discontinuation of chemotherapy is known.

The cardiac-surveillance strategy developed for this trial represents the first such prospective algorithm for women receiving trastuzumab. On the basis of this experience, we believe patients with normal baseline cardiac function can have a single re-examination of LVEF after 16 weeks of trastuzumab-vinorelbine treatment. Those patients without cardiac symptoms or without substantial declines in LVEF may be observed thereafter, without frequent cardiac surveillance.

Patients who had tumors that were HER2-positive were eligible for this trial by virtue of documented gene amplification (ie, FISH-positive) or marked surface expression by IHC 3+-positive because we recognize that trastuzumab efficacy appears limited to those patients with clear HER2 overexpression.[8] In our trial, equal response rates were noted among patients eligible by virtue of IHC 3+-positive (n = 44) and FISH-positive (n = 10) tests, indicating that either test as performed by participating centers is appropriate for selecting patients for trastuzumab-based therapy. The optimal testing method for selecting patients for trastuzumab-based therapy remains uncertain.

Predictive markers for response to trastuzumab-based treatment—aside from tumor HER2 status—are not well characterized. Previous research has indicated that high HER2 ECD levels were associated with greater likelihood of tumor response among 30 patients receiving trastuzumab-docetaxel treatment and that changes in HER2 ECD correlated with tumor response.[14] We prospectively evaluated the utility of serum HER2 ECD as a predictor of response during cycle 1 of trastuzumab-vinorelbine therapy. We did not find that baseline HER2 ECD was a predictor of response or that change in HER2 ECD predicted response. This lack of association was due principally to declines in HER2 ECD, even among patients with stable disease. This indicates that trastuzumab therapy may be associated with clearance of HER2 ECD, as measured by immunoassay. In contrast, lack of decline in HER2 ECD was a predictor for tumor progression after cycle 1 of therapy. At present, we do not believe that measurement of HER2 ECD is sufficiently reliable for determining response to trastuzumab-based therapy, although additional studies that involve larger numbers of patients for longer periods of time are warranted.

The optimal trastuzumab-based chemotherapy regimen is not known. Our data support the use of trastuzumab and vinorelbine as a safe, well-tolerated, and effective first-line treatment for women with HER2-positive metastatic breast cancer. The regimen is currently being compared to taxane-based trastuzumab regimens in the metastatic setting and is under evaluation as preoperative therapy for women with HER2-positive stage II or III breast cancer.

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Circulating Tumor Cells in HER-2 Positive Metastatic Breast Cancer Patients treated with Trastuzumab and Chemotherapy

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INTRODUCTION

Although 90% of invasive breast cancers appear limited to the breast and regional lymph nodes at diagnosis, occult micrometastases are common, and up to 40% of these patients will recur without systemic therapy[1]. While tumor size and nodal status are the best predictors of systemic recurrence, recent data suggests that analysis of blood and bone marrow for micrometastases may give additional prognostic information [2,3]. Detection of micrometastases from blood has the advantage of lower morbidity than biopsy and allows serial sampling during the treatment course. Traditional detection methods using immunohistochemistry may have limited sensitivity in detecting very rare circulating cancer cells. More recently, analysis of gene expression using reverse transcription polymerase chain reaction (RT-PCR) has been employed for detection of micrometastases. While these methods have increased sensitivity, and allow the detection of as few as one epithelial cell in 10^7 mononuclear blood cells, specificity remains an important issue[4].

The combination of paclitaxel chemotherapy with the anti-HER-2 antibody, trastuzumab (Herceptin) has been shown to increase survival in women with HER-2 positive metastatic breast cancer[5]. Our group has evaluated the combination of Herceptin and vinorelbine and found encouraging response rates with low toxicity[6]. We serially evaluated serum HER-2 levels and circulating tumor cells in HER-2 positive, metastatic breast cancer patients treated on a Phase II clinical trial of trastuzumab and vinorelbine. In order to perform tumor cell detection, we combined immunomagnetic selection with a novel semiquantitative RT-PCR based method. The goal of this study was to develop a sensitive and specific method for circulating tumor cell detection in HER-2 positive

breast cancer, and to validate its ability to track disease response and progression during therapy.

MATERIALS AND METHODS

Study Design

Patients with HER-2-positive and metastatic breast cancer were treated on a first-line phase II multicenter study of trastuzumab and vinorelbine. HER-2-positive cancers were scored as 3+ positive by immunohistochemistry or were positive for gene amplification by Fluorescent In-Situ Hybridization (FISH). Treatment protocol and clinical response data have been published previously [7]. In selected centers, with available infrastructure, peripheral blood samples were collected prior to therapy (week 0), and at weeks 2, 3, 5 and 8 of the first cycle and at every restaging event thereafter. Clinical restaging, using RECIST criteria[8], occurred every 8 weeks. All patients provided informed consent for treatment and sample collection. Several patients who progressed on therapy were enrolled in other trastuzumab-containing trials conducted at our institution and had serial samples collected. These patients were included in the analysis.

Cell collection

At each time-point, 8 mL of peripheral blood were collected in cell preparation tubes (CPT, Becton-Dickinson), mixed well and centrifuged within 2 hours. Two to 3 mL of plasma were recovered and kept frozen at −80. The mononuclear cell (MNC) fraction was collected, washed in PBS, counted, resuspended in a freezing media

containing 10% DMSO and 20 % fetal bovine serum (FBS) and frozen in a rate-controlled freezer (Forma, Scientific). Frozen samples were kept at –140.

Immunomagnetic selection and RNA extraction

Positive selection for tumor cells was performed using magnetic beads coated with a 40 μg/ml suspension of BerEP4, an anti-epithelial antibody directed against the glycoprotein EPCAM (Dynabeads, Epithelial Enrich, Dynal). Prior to analysis, cells were rapidly thawed in a 37° water bath, washed in RPMI with 20% of FBS and resuspended in 8 ml of chilled PBS solution containing 0.6 g of sodium citrate and 1% FBS. Eighty microliters of coated beads were added to the cell suspension. The cell and bead mixture was incubated for 30 min with rotation and the beads recovered using a magnet and washed to reduce non-specific binding. RNA extraction from the bead-attached cellular fraction was performed using Rneasy MiniKits (Qiagen). PolyA (30μg/ml) was added to the lysis buffer as a carrier. RNA was resuspended in 50 μl of RNAse-free water. Given the low amount of RNA obtained after immunomagnetic selection, quantification was not possible. It was estimated that an epithelial cell enrichment of more than 3 logs is obtained with positive immunomagnetic bead selection[9].

Cytokeratin-19 (CK-19) detection

CK-19 and B2 microglobulin (to control for RNA integrity) were amplified and detected using a platform developed by Abbott Laboratories. For each marker, a premix containing specific primers and probes was used. CK-19 primers were designed to span introns and avoid hybridization with pseudogenes. Amplification was performed using unit

dose vials containing buffer, nucleotides and a thermostable polymerase with reverse transcriptase activity. Prior to amplification, the oligonucleotide mix, Mn^{++} and 5 uL of RNA were added to the unit dose vial. Thermal cycling conditions were as follows: 60 minute incubation at 60° to permit reverse transcription, followed by 94° for 40 seconds and 58° for 1 minute for 45 cycles. After cycling was complete, the temperature was increased above the melting point of the amplification product and quickly lowered to 12 °C, to allow the detection probe present in the mix to anneal to dissociated product strands and generate a detectable amplicon-probe complex. This complex constitutes the product that is detected by Microparticle Enzyme Immunoassay (MEIA). In the LCx® Analyzer (Abbott Labs, Inc), aliquots of the amplification product and microparticles were transferred to a reaction cell. Microparticles bind the amplicon-probe complex as well as non-hybridized probes. The reaction mixture was then transferred to a glass fiber matrix to which the microparticle complexes bind irreversibly. A wash step is necessary to remove non-extended primers and non-specific products. The bound microparticle complexes were then incubated with the alkaline phosphatase conjugate, which binds to the adamantane. This antibody conjugate is detected by addition of the substrate, 4-methylumbelliferyl phosphate, which is dephosphorylated by alkaline phosphatase to produce a fluorescent molecule, 4-methylumbelliferone, and measured by the MEIA optical assembly. The change in fluorescent energy serves as the reported value expressed in counts/sec/sec (c/s/s).

Sensitivity and specificity of tumor cell detection

To establish the specificity of tumor cell detection, peripheral blood from 40 healthy female blood donors was collected from the Brigham and Women's Hospital

Blood Donor Center, after informed consent. The sensitivity was established by spiking MDA-MB-361 (HER-2 amplified) cells into whole blood from healthy donors, collected in CPT and processed in a similar fashion to patient samples. All samples were analyzed in duplicate or in triplicate when results were discordant.

Shed Extracellular Domain of HER-2 (HER2/ECD)

HER2/ECD was measured using a sandwich immunoassay developed by Oncogene Science, Inc. In brief, 200 μL of serum were incubated in the well coated with the capture antibody, reacting with the detector antiserum. The amount of detector antibody bound to antigen was measured by binding it with a streptavidin/horseradish peroxidase conjugate. All samples were analyzed in duplicate. The inter-assay coefficient of variation was 3.1%. The cut-off value for a positive result was set at 20 ng/ml.

Statistics

The Fisher exact Test was used for all comparisons.

RESULTS

Sensitivity

In 20 experiments, the presence of 10 MDA-MB-361 breast cancer cells spiked in 8 ml of whole blood was always detectable. CK-19 signal by LCx® consistently measured 900-1300 c/s/s for 10 breast cancer cells spiked into blood. RNA dilution assays allowed the detection of 0.01 cell equivalent of CK-19 (Figure 1). No

amplification was obtained with omission of the reverse transcription step, confirming that detection of genomic DNA did not occur.

Specificity

Without tumor cell enrichment, detection of CK-19 in the blood of healthy controls occurred frequently and immunomagnetic selection was essential to assure the specificity of detection (Figure 2). Blood from 40 healthy donors was analyzed by LCx® after mononuclear cell isolation, immunomagnetic selection and RNA extraction. for B2MG and CK-19 transcripts. In 35 cases (87.5%), LCx® values of 10-50 c/s/s were obtained in the duplicate analyses of CK 19 transcripts . In one case, a value > 100 c/s/s was obtained in the duplicate analysis. In 4 cases, duplicate results were discordant (one value >100, one value <100 c/s/s) and a third analysis was performed. Based on these results, a positive result for CK-19 detection was defined as the presence of duplicate values >100 c/s/s. When the duplicates were discordant, values > 100 c/s/c in 2 out of 3 analyses were considered a positive result. B2MG was detectable in all cases. With this definition, CK-19 was detected in 2 (5 %) healthy controls, with counts averaging 479 c/s/s.

CK-19 Signal Detection and Disease Characteristics in Metastatic Breast Cancer Patients on Trastuzumab and Chemotherapy

One hundred ninety-seven serial samples on 27 patients were analyzed by LCx®. Baseline samples were available in 25 patients. At baseline, 11 (44 %) patients were CK-

19 positive, with a median count of 1034 c/s/s (range 480-1199). In 14 patients, the CK-19 value was in the negative range (median result 17.7 c/s/s, range 10.7-23.4)

All patients with tumor cells detectable in circulation had liver metastases (p=0.00002). Presence of CK-19 signal at baseline was associated with visceral disease (p=0.02) but not with other clinical variables including number of metastatic sites, and ER status (Table1). All patients included in the study had tumors, which were HER-2 3+ by immunohistochemistry or amplified for the HER-2 gene by fluorescent in-situ hybridization.

Serial Analysis of CK-19 Signal in Metastatic Breast Cancer Patients on Trastuzumab and Vinorelbine

The overall response rate for the combination of trastuzumab and vinorelbine was 68%[7]. Of the twenty-three patients who had baseline and serial assessment of CK-19 through at least one cycle of therapy, 20 had complete response, partial response or stable disease after 8 weeks of trastuzumab and vinorelbine. , Three patients progressed at the first cycle of therapy. None of the eleven patients with CK-19 signal present at baseline had disease progression at first restaging. In all of these patients, CK-19 signal declined during the first two cycles of therapy (Figure 3). Progression occurred in three patients during the first cycle of therapy, all of them CK-19 negative at baseline. In one of three, the CK-19 signal became consistently detectable five weeks prior to restaging, when the patient developed lung and liver disease progression (Figure 4, patient 15). The other two patients who progressed at first re-staging did not demonstrate CK-19 signal at any timepoint (Figure 4).

With subsequent cycles of trastuzumab and vinorelbine, detection of CK-19 signal occurred in three cases (Figure 4, patients 7, 10 and 20). One patient was removed from therapy for toxicity in complete response - further follow-up is not available. The other two patients had CK-19 detectable at a single timepoint, one or two cycles before disease progression (one patient progressed in liver and lung; the other in brain).

In patients with disease progression whose CK-19 signal remained negative (n=7) four patients developed brain metastases without evidence of systemic recurrence. The others had lung and liver progression (Figure 4).

Three patients developed CK-19 signal after progression on trastuzumab and vinorelbine. These patients were enrolled in a second line trastuzumab trial and had samples collected in the course of treatment. In all cases, these patients progressed without initial response to the second regimen (figure 4, patient numbers 21, 22 and 23).

Comparison of HER 2/ECD and Tumor Cell detection

Data on HER-2/ECD and CK-19 detection was available in twenty-four patients. Twenty-three had baseline samples. Serum HER-2/ECD was elevated at baseline in 12/23 patients (52%) with a median value of 63.5 ng/ml (range 21.5-616 ng/ml). While HER-2/ECD was seen in patients with and without CK-19 signal, HER-2/ECD levels over 50 ng/ml (median value 228 ng/ml) were only seen in patients who were positive for CK-19 signal ($p=0.008$). In all of these patients, CK-19 signal and HER-2/ECD declined in a similar fashion. In two of the three patients with samples collected after progression on trastuzumab and vinorelbine, both serum HER-2/ECD and CK-19 became elevated with liver disease progression (Figure 5).

DISCUSSION

Detection of micrometastases in bone marrow or blood may be a useful marker of early recurrence, or predict response to therapy in patients with breast cancer. Several studies suggest that the presence of breast cancer cells in the bone marrow is associated with a worse outcome [10 11 12]. In addition to the prognostic impact of cytokeratin-positive bone marrow cells at diagnosis, [13] a similar effect on prognosis after adjuvant chemotherapy[14], and at disease relapse[15] has been demonstrated. Furthermore, CK-19 positive cells present in the bone marrow were shown to have clonogenic potential, suggesting these cells are not merely 'innocent bystanders'[16].

Detection of tumor cells in the peripheral blood has several advantages over bone marrow biopsy, including lower morbidity of tissue sampling and the opportunity for serial monitoring of disease. However, this field of study has been limited by the technical challenge of detecting small numbers of malignant cells in the peripheral blood. Based upon published studies, it is estimated that circulating tumor cells (CTCs) are present at a rate of 1-100 malignant epithelial cells per ml (10^6) of mononuclear cells[17]. Highly specific and sensitive methods are necessary to detect these 'rare events'.

Reverse transcriptase polymerase chain reaction (RT-PCR) is a promising method for detection of microscopic disease in peripheral blood, due to its high sensitivity. Cytokeratins are abundant in the vast majority of breast cancer cells [18] and frequently used to detect breast cancer cells in bone marrow or peripheral blood. In *in vitro* model systems, RT-PCR for Cytokeratin 19 can detect 1 tumor cell in 10^6-10^7 mononuclear cells[19 20 21]. Other markers, such as mammaglobin[22], EGF-R[23], CEA[24], BHCG[25],

Maspin[26], HER-2 [27] and telomerase[28] have been also used. Although most markers demonstrate similar sensitivity for detection in model systems, available data from patient samples show variable rates of detection, from 0-54% in early stage patients to 13-84% in metastatic disease. Furthermore, many of the targets previously reported as specific were later found to be present at low frequency in the blood or bone marrow of healthy controls [4] [29]. The detection of RT-PCR signal in normal blood is attributed to the amplification of illegitimately transcribed genes in hematopoietic cells, contaminating non-malignant epithelial cells, and the amplification of pseudogenes from contaminating genomic DNA. Hence, RT-PCR-based cancer cell detection in peripheral blood samples must address the possibility to false-detection due to contaminating CK-19 transcripts in normal blood cells.

This study utilized immunomagnetic selection of circulating epithelial cells to address the specificity of tumor cell detection using CK-19. Differences in the efficiency of mononuclear cell depletion could explain the presence of positive results in normal controls [30]. Although this may have confounded our analysis, positive values in patient samples tended to be higher and similar to experimentally spiking malignant epithelial cells into blood samples. In addition, the consistency CK-19 signal detection over time, within the same patient, and the association of these findings with response to therapy suggests that this signal was derived from cancer cells, and not from contaminating mononuclear cells.

As further validation of our technology, the extracellular domain of the HER-2 protein (HER-2/ECD) was determined in parallel. HER-2/ECD has been detected in the serum of 20-50% of patients with metastatic breast cancer [31] [32]. HER-2/ECD is

correlated with a worse prognosis [33] [34] [35] [36] and is associated with disease burden. The rate of decline of serum HER-2 appears to predict response in trastuzumab treated patients, although this relationship is complex in the context of antibody treatment [37] [38].

We studied a group of patients with HER-2 positive metastatic breast cancer, reasoning that the likelihood of circulating tumor cells is higher in later stage patients with HER-2-postive breast cancer, which tend to present with extensive metastases [33]. In addition, the HER-2-positive group is likely to express CK-19 and be more biologically homogeneous than an unselected cohort of patients[39]. Furthermore, this assay was performed during a controlled clinical trial to ensure reliable data collection and measures of disease response. We detected CK-19 in 11 of 25 (44%) of women with metastatic HER-2-positive breast cancer, prior to therapy. CK 19 detection was strongly associated with the presence of liver metastases ($p=0.00002$). In responding patients, CK 19 declined over the first two cycles (Figures 3 and 4). All patients with HER2/ECD> 50 ng/ml had CK-19 signal detectable ($p=0.008$). In addition, patients who developed high levels of HER2/ECD at progression were also positive for CK-19 detection . Our results support the concept of molecular tumor cell detection in the circulation of patients with metastatic breast cancer, and the development of this surrogate to monitor treatment response.

Studies reporting serial monitoring of circulating tumor cells in patients undergoing systemic therapy support the general conclusions of our work. For instance, a study employing immunomagnetic selection of EPCAM positive cells, flow cytometry and immunohistochemistry reported on eight patients with breast cancer, treated in different ways and examined for 1 to 10 months at various intervals. In this group, the

number of epithelial cells detected was always parallel to the clinical status of the patient [40]. In a second study of 15 patients with metastatic breast cancer treated with various regimens, detection of CTC with competitive PCR and IHC for CK-19 reflected the treatment outcome in 68% and 57% of cases, respectively [41].

This study monitored the presence of CTC in patients with HER-2 positive metastatic breast cancer, serially monitored during a controlled clinical trial. We show that detection of CTC, is feasible and these cells are not detected randomly, but have a consistent pattern in each patient. While correlating generally with disease burden as measured by response and progression, sensitivity of CTC detection is low, either due to limitation of the technique or to underlying differences in the biology of metastasis in different patients. In our study, liver metastasis correlated highly with detection of CTC. It's not clear whether this reflects disease burden or a biologic feature of the tumor, as detection of CTC was not more common in patients with more disease sites involved. Although circulating tumor cells are rare, even in the presence of metastatic disease, further study may reveal more about the pathophysiology of circulating cells shed from tumors. This in turn, may lead to improved methods of tumor cell detection in peripheral blood. Detection of tumor cells in blood could provide an inexpensive and minimally invasive way to monitor the treatment response of patients with metastatic breast cancer.

Acknowledgments

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Figure Legends

Figure 1. Sensitivity of CK-19 detection. Five MDA 361 cells were spiked in 8 ml of peripheral blood from normal donors and selected with magnetic beads. RNA from the bead-attached population was extracted, submitted to 1:10 serial dilutions and expressed as cell equivalents. Error bars for each dilution point were calculated as the mean of 20 experiments, plus or minus one standard deviation.

Figure 2. Detection of CK-19 positive cells in normal blood and in blood with 10 CK-19 positive cells spiked, before and after immunomagnetic selection (IMS). Error bars for each determination were calculated as the mean of 20 experiments, plus or minus one standard deviation.

Figure 3. Serial analysis of CK-19 at baseline, and at re-staging. C1, re-staging after cycle 1 of trastuzumab and vinorelbine therapy; C2, re-staging after cycle 2 of trastuzumab and vinorelbine. PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease.

Figure 4. CK-19 analysis in patients who completed at least one cycle of therapy. Black squares, CK-19 positive. White squares, CK-19 negative. Dotted lines (....) indicate a switch to new regimen. PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; W, week; C, cycle.

Figure 5. Serial evolution of CK-19 and HER2/ECD in the patients with samples collected after first progression. PD, progressive disease.

FIGURE 1

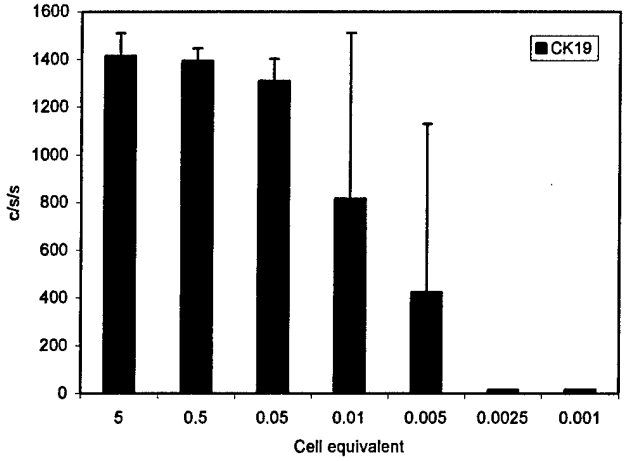
1600
1400
1200
1000
800
600
400
200
0
c/s/s
CK19
5
0.5
0.05
0.01
0.005
0.0025
0.001
Cell equivalent

FIGURE 2

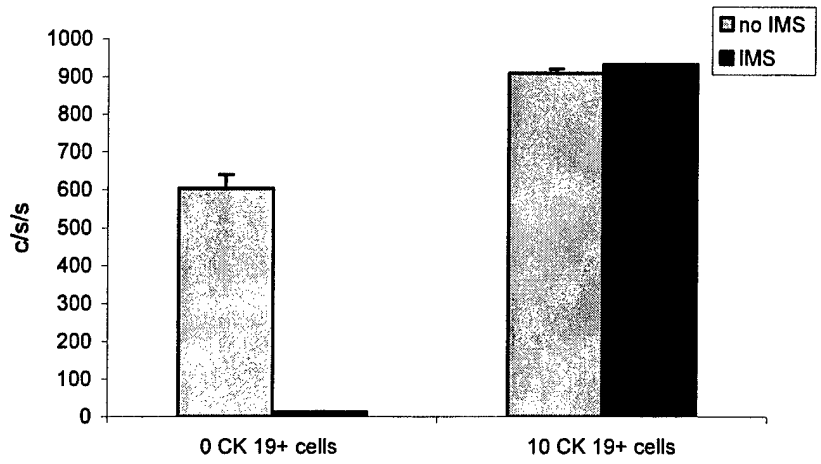
no IMS
IMS
1000
900
800
700
600
500
400
300
200
100
0
c/s/s
0 CK 19+ cells
10 CK 19+ cells

FIGURE 3

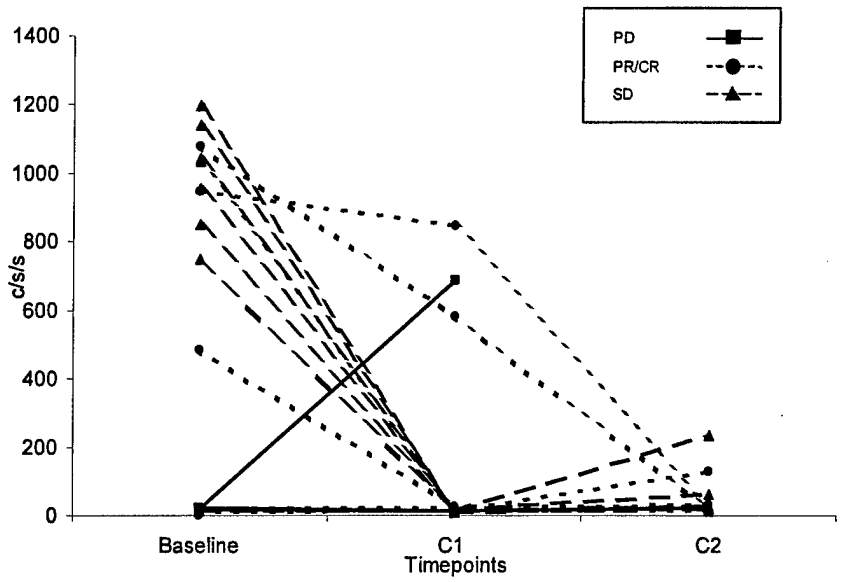
PD
PR/CR
SD
1400
1200
1000
800
600
400
200
0
c/s/s
Baseline
C1
C2
Timepoints

FIGURE 4

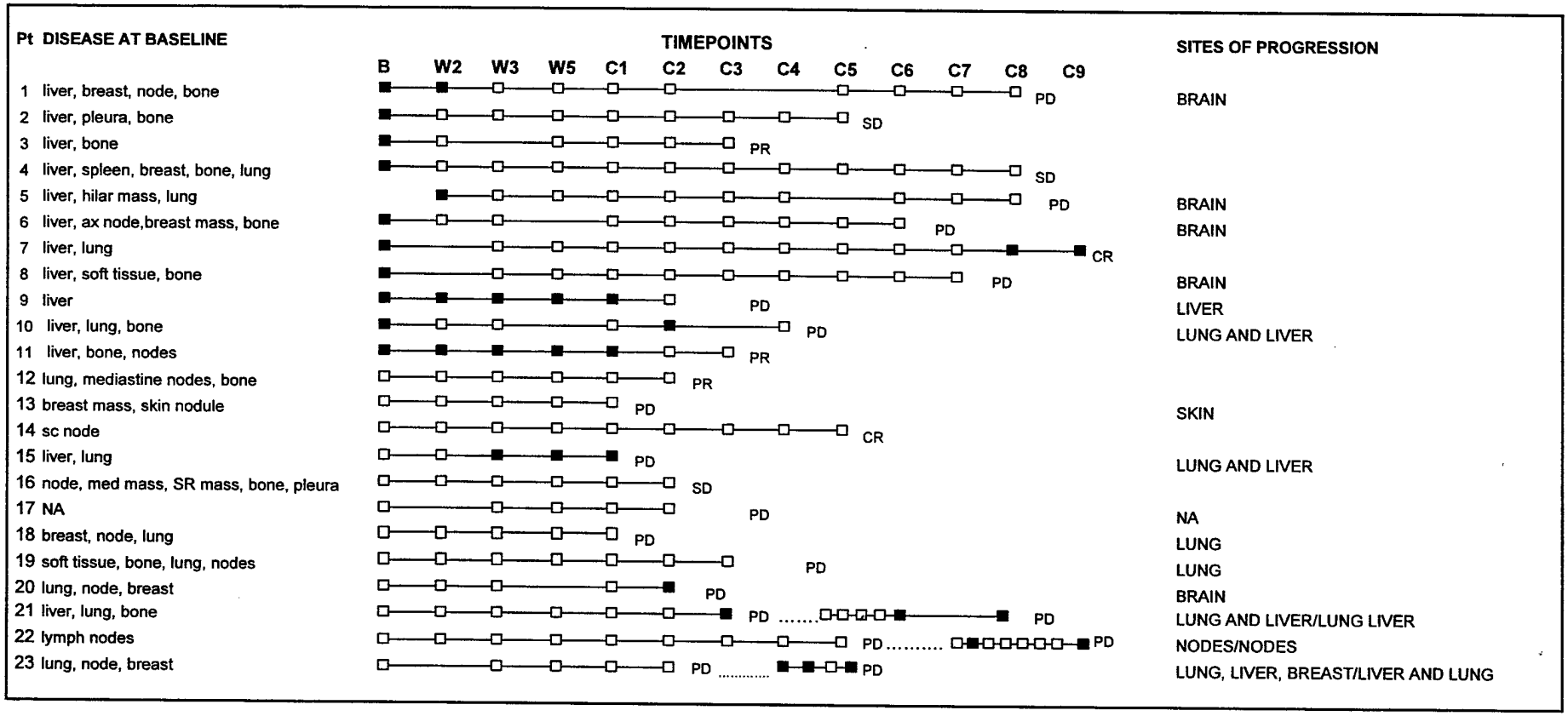
Pt DISEASE AT BASELINE
TIMEPOINTS
B W2 W3 W5 C1 C2 C3 C4 C5 C6 C7 C8 C9
SITES OF PROGRESSION
1 liver, breast, node, bone
PD
BRAIN
2 liver, pleura, bone
SD
3 liver, bone
PR
4 liver, spleen, breast, bone, lung
SD
5 liver, hilar mass, lung
PD
BRAIN
6 liver, ax node,breast mass, bone
PD
BRAIN
7 liver, lung
CR
8 liver, soft tissue, bone
PD
BRAIN
9 liver
PD
LIVER
10 liver, lung, bone
PD
LUNG AND LIVER
11 liver, bone, nodes
PR
12 lung, mediastine nodes, bone
PR
13 breast mass, skin nodule
PD
SKIN
14 sc node
CR
15 liver, lung
PD
LUNG AND LIVER
16 node, med mass, SR mass, bone, pleura
SD
17 NA
PD
NA
18 breast, node, lung
PD
LUNG
19 soft tissue, bone, lung, nodes
PD
LUNG
20 lung, node, breast
PD
BRAIN
21 liver, lung, bone
PD
PD
LUNG AND LIVER/LUNG LIVER
22 lymph nodes
PD
PD
NODES/NODES
23 lung, node, breast
PD
PD
LUNG, LIVER, BREAST/LIVER AND LUNG

FIGURE 5

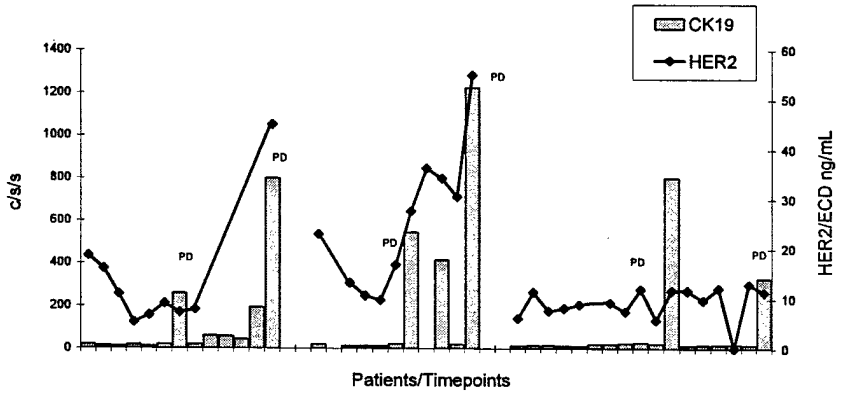
CK19
HER2
c/s/s
HER2/ECD ng/mL
Patients/Timepoints
PD
PD
PD
PD
PD
PD
0
200
400
600
800
1000
1200
1400
0
10
20
30
40
50
60

Table 1. Patient characteristics and CK-19 at baseline

Patients	CTC[a] positive (n=11)	CTC negative (n=14)	p value[b]
Receptor Status			
ER/PR positive	8	5	0.11
ER/PR negative	3	7	
Unknown		2	
Number of disease sites			
≤2	3	4	0.33
> 2	8	8	
Unknown		2	
Location of disease			
Visceral	11	7	0.02
Non Visceral	0	5	
Unknown	0	2	
Liver	11	1	0.00002
Not liver	0	13	

[a] Circulating tumor cells, determined by LCx for CK-19
[b] Significance tested by Fisher's Exact Test